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NUCLEAR MAGNETIC RESONANCE STUDIES OF PHOSPHOPROTEINS

by

© HARM JAN VOGEL

A THESIS
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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
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ABSTRACT

^{31}Phosphorus Nuclear Magnetic Resonance (^{31}P-NMR) has recently become a popular tool to study metabolism *in vivo*. Here it is demonstrated that additional metabolic information can be obtained by similar studies using whole cell extracts. The resonances in such ^{31}P-NMR studies can be readily assigned on the basis of their chemical shifts. However, pH titration and metal binding studies of ATP and ADP methylene- and fluoroanalogues have shown that this parameter is not always easily interpretable in terms of molecular detail. Thus although the chemical shifts usually provide reliable information about the chemical nature of the linkage that is present in a phosphoprotein, other parameters must be measured to abstract additional molecular information. This dissertation is concerned mainly with the measurement and analysis of the linewidth and the pH titration behaviour of a variety of phosphoproteins as measured by ^{31}P-NMR spectroscopy.

A first group of phosphoproteins that has been studied comprises those in which the phosphorus moiety is involved in metal ion complexation, with hen egg-yolk phosvitin as a prototype. ^{31}P-NMR pH titrations, together with assessment of the accessibility of aromatic residues as determined by photo-laser CIDNP ^{1}H-NMR experiments, indicated that this protein has an unusual tertiary structure which is primarily

stabilised by salt linkages and hydrogen bond interactions involving the phosphoryl moieties.

A second group of phosphoproteins includes all enzymes and proteins whose activity is controlled by reversible phosphorylation. Analysis of the frequency dependence of the linewidths measured in ^{31}P-NMR spectra obtained at five different magnetic field strengths has shown that the regulatory sites of glycogen phosphorylase *a* and hen egg-white ovalbumin have some flexibility. A similar site on polymerized rabbit skeletal tropomyosin also showed mobility. It was further demonstrated that all these residues could be titrated, thus indicating their accessibility to solvent. Since similar behaviour was recently reported for regulatory phosphorylation sites of troponin T and myosin light chains, it appears that flexibility and titrability are a common feature of such sites. The phosphate previously thought to be covalently attached to bacterial conjugative pili was shown to be rigidly associated phospholipids.

A third group of phosphoproteins comprises all enzymes with covalent phosphoryl intermediates. These studies have indicated that such entities are generally immobilized in the active sites of the enzymes. Other reports had already demonstrated that such residues generally do not titrate. In particular, studies on the N-3 phosphohistidine residue of *E. coli* succinyl-CoA synthetase have provided insight into the complex mechanism of this multisubunit enzyme. Evidence has been obtained for conformational changes of this residue that are induced by addition of other substrates. Moreover it was shown that the active sites of this enzyme do not act independently but act in a catalytic cooperative way. Further support for such behaviour was obtained from inhibition experiments and from studies making use of hybrids of the enzyme containing chemically modified inactive subunits. Moreover the conformational flexibility of the enzyme molecule, necessary to provide [illegible] intrasubunit communication, was deduced from ^{1}H-NMR studies. Proteolytic- and thermal inactivation studies on the mechanistically related enzyme ATP-citrate lyase showed that this enzyme undergoes conformational changes by binding of a wide variety of phosphorylated compounds. Nucleotide binding site mapping studies have indicated the determining role of the phosphoryl moieties of ligands in the high precision recognition between this enzyme and its ligands.

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LIST OF ABBREVIATIONS

ADPβF	Adenosine-5'-O-(2-fluorodiphosphate)
ADPβS	Adenosine-5'-O-(2-thiodiphosphate)
AMPCP	α,β Methylene Adenosine-5'-diphosphate
AMPCPP	α,β Methylene Adenosine-5'-triphosphate
AMPF	Adenosine-5'-O-fluoromonophosphate
AMPPCP	β,γ Methylene Adenosine-5'-triphosphate
AMPPNP	Adenylyl-5'-yl-Imidodiphosphate
ATPαS	Adenosine-5'-O-(1-thiotriphosphate)
ATPβS	Adenosine-5'-O-(2-thiotriphosphate)
ATPγF	Adenosine-5'-O-(3-fluorotriphosphate)
ATPγS	Adenosine-5'-O-(3-thiotriphosphate)
CD	Circular Dichroism
CIDNP	Chemically Induced Dynamic Nuclear Polarization
CSA	Chemical Shift Anisotropy
DSS	4,4 dimethyl-4-silapentane-1-sulfonic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetate
hisP	Histidine-phosphate
HPr	Bacterial heat resistant phosphoryl carrier protein
NEM	N-ethylmaleimide
Pi	Inorganic phosphate
PEP	Phosphoenolpyruvate
PMSF	Phenyl methyl sulfonyl fluoride
ppGpp	Guanosine-3'-diphosphate-5'-diphosphate

PTS	Bacterial phosphotransferase sugar transport system
PyrP	Pyridoxal phosphate
SCS	Succinyl-CoA synthetase
SDS	Sodium dodecyl sulfate
SerP	Serine phosphate
Succ-P	Succinyl phosphate
TCA	Tricarboxylic acid
TPCK	L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone

CHAPTER 1

INTRODUCTION

1.1 Chemistry of phosphorus compounds

The element phosphorus is a common and key component of many biological molecules. Although it is stable in a wide variety of oxidation states, the only one found in living matter is +5 and no net changes in the redox state have been reported [1]. Under these conditions the phosphorus atom behaves as an electrophile and is capable of undergoing attack by a wide variety of nucleophiles. The predominant form in which it is found is the phosphate group where the central phosphorus atom is surrounded by four electronegative oxygen atoms in a tetrahedral arrangement. As a second row element of the periodic table, it can form a fifth bond by putting additional electrons into unfilled d orbitals [2]. Such trigonal bipyrimidal pentacoordinate structures, although chemically stable, have never been found as products of enzymatic reactions but have often been proposed as intermediates in associative mechanisms for phosphoryl transfer enzymes [3]. Dissociative mechanisms have been suggested as well and these proceed via a planar trigonal metaphosphate intermediates. Recent studies on the stereochemistry of enzymatic phosphoryl transfers indicate that most proceed via "in-line attack". Thus the associative mechanism is more likely to occur [3,4]. Active site NMR distance measurements have largely confirmed this by showing that the distances between nucleophile and electrophilic phosphorus atom are small enough (3 Å) to allow for such a mechanism [5]. In a few cases some "dissociative character" maybe involved [5], but shearing of protein domains which constitute the binding sites for the two substrates could also "close the gap".

We can write two resonance forms for phosphoric acid, the parent compound of all biological molecules.

$$^{-}O - {}^{+}P(OH)_3 \longleftrightarrow O \quad P(OH)_3$$

The first form emphasises the electrophilicity of the phosphorus atom. The other form indicates the double bond which comprises a o as well as a d-p component and is thus not a "normal" double bond [1,2]. This bond does not alter the tetrahedral geometry of the substituents around the phosphorus atom. Complexations of cations to the oxygen atoms or replacement of one of these atoms by those of another element with different electronegativity will result in changes in the above mentioned pattern of σ and π-bonding [6]. Similarly bond-geometry-distortions, such as those imposed in cyclic ring systems, may have similar effects on the electron distribution.

1.2 Biological phosphate compounds

Phosphoric acid exists at physiological pH mainly as the dianion. Similarly all phosphomonoesters ($RO-PO_3^{2-}$) and phosphodiesters [$(RO)_2$-PO_2^-] and most other phosphate compounds are all anionic at physiological pH. Such negative charges give rise to electrostatic repulsion, which make phosphate esters resistant to hydrolysis. Either protonation or complexation with metal cations can facilitate attack by water and other nucleophiles. Metal ions are more effective than protons for this function since they can exist at high concentrations in neutral solutions and usually serve as multidentate coordinators. Thus it is not surprising that "essential" arginine residues line the active sites of phosphoryl transfer enzymes [7] and that metal ions (i.e. Mg^{2+} and Mn^{2+}) are known to be a necessary requirement for catalysis in most of these systems [5,8]. Both arginines as well as metal ions can serve as a template for recognition and orientation of phosphorylated substrates and products but may also enhance the electrophilicity of the phosphorus atom, thus rendering it a better substrate for nucleophilic attack.

Virtually all glycolytic pathway intermediates and most coenzymes and energy carriers (i.e. ATP) exemplify the ubiquitous role of phosphomonoesters and phosphodiesters in biological systems. Well known examples of monoester compounds are phosphorylated sugars and the amino acid phosphoserine. Phosphodiesters appear mainly as the pyrophosphate group (R-O-PO_2^--O-PO_3^{2-} as in ATP, or R-O-$(PO_2^-)_2$-O-R as in CoA) but do also appear in the internucleotide monophosphodiester bond that is the structural backbone of DNA and RNA ($R\text{-}O\text{-}PO_2^-\text{-}O\text{-}R$); this similar linkage is also found in a variety of phospholipids [9].

Although all biologically occurring phosphates have four substituents, the neighbouring atom does not necessarily have to be an oxygen. For example, low molecular weight phosphoramidates like phosphocreatine and phosphoarginine ($R\text{-}N\text{-}PO_3^{2-}$) function as a reservoir for "high energy" phosphoryl groups in skeletal and invertebrate muscle respectively. Also, N-3 or N-1 phosphohistidine residues have been identified in the alkaline hydrolysates of several enzymes [10]. Such compounds have a high free energy of hydrolysis, a property they share with the acylphosphates ($R\text{-}CO\text{-}OPO_3^{2-}$) making both classes of compounds equally suited for a role as enzymatic intermediate phosphoryl group carrier, while conserving energy. X-ray crystallographic studies of phosphoramidates [11] indicate that such structures deviate even more than the phosphomono- and diesters [12] from the tetrahedral structure of orthophosphate. This will probably facilitate nucleophilic attack on the phosphorus atom in such molecules.

For the sake of completion it should be mentioned here that recently a thiophosphate ($R\text{-}S\text{-}PO_3^{2-}$) linkage has been found [13] and that phosphonates ($R\text{-}CH_2\text{-}PO_3^{2-}$) have recently been elegantly demonstrated *in vivo* by ^{31}P-NMR of an amoeba [14]. Although such linkages rarely occur in nature, experimentalists have made use of a whole variety of synthetic phosphate analogues containing such bonds. Their properties differ from naturally occurring compounds and they can be used to advantage to provide insight in catalytic and regulatory mechanisms [15].

In this dissertation several biological phosphorylated compounds and their analogues have been studied in order to supply background necessary for understanding the more complex phosphoproteins (see Chapter 2, 3 and 8). Moreover, the role played by the phosphate moieties of coenzymes in recognition by macromolecules is explored in Chapter 12.

1.3 Enzymatic transfer of the phosphoryl group

Enzymes catalyzing reactions involving transfer of phosphoryl groups make up a large class of group transfer reactions that are central to metabolism. On the basis of the kind of reactions that they carry out they can be readily subdivided into five classes (see Table 1.1). Examples of well-characterised enzymes are given for each class. Both phosphatases and diesterases use water as a substrate. Kinases and mutases use another substrate or another group on the same substrate as the nucleophile. The mechanistically common feature of these four classes is that all catalyse transfer of a phosphoryl group ($-PO_3^{2-}$), whereas the class of phosphorylases catalyses the transfer of a phosphate group ($HO-PO_3^{2-}$).

Table 1.1

Classification of enzymes catalysing phosphoryl group transfer[1]

1. Phosphatases	$RO\text{-}PO_3^{2-} + H_2O \rightleftharpoons R\text{-}OH + HO\text{-}PO_3^{2-}$. Alkaline-, acid, glucose-6-phosphatases; ATP-ases; inorganic pyrophosphatase; protein phosphatases.
2. Phosphodiesterase	$RO\text{-}PO_2^{-}\text{-}OR' + H_2O \rightleftharpoons R'\text{-}O\text{-}OPO_3^{2-} + R'OH$. Ribonucleases; DNA-ses; 3'5' cAMP phosphodiesterase.
3. Phosphokinases	$R\text{-}XPO_3^{2-} + R'Y \rightleftharpoons R'\text{-}Y\text{-}PO_3^{2-} + RXH$. (1) Kinases: hexo-, phosphofructo-, pyruvate-, creatine-, arginine-, adenylate-, acetate-, phosphoglycerate-, nucleoside diphospho-, protein kinase; phospho- transacetylase, PEP dependent hexose-P transport system. (2) Nucleotidyl transfer enzymes: DNA and RNA polymerases, galactosyltransferase. (3) Pyrophosphoryl transferring enzymes: PRPP synthetase, PEP-syntase, pyruvate-P-dikinase. (4) Ligases depending on phosphoryl group cleavage: succinyl-CoA synthetase, ATP-citrate lyase, acetyl-CoA synthetase, aminoacetyl-tRNA synthetase, adenylosuccinate synthetase, glutamine synthetase.
4. Phosphomutases	$HO\text{-}R\text{-}O'\text{-}PO_3^{2-} \rightleftharpoons PO_3^{2-}\text{-}O\text{-}R\text{-}O'H$. Phosphogluco- and phosphoglycerate mutase.
5. Phosphorylases	$R\text{-}O\text{-}R' + HO\text{-}PO_3^{2-} \rightleftharpoons R\text{-}O\text{-}PO_3^{2-} + R'OH$. Polynucleotide and glycogen phosphorylase.

[1] Data obtained from references 1, 3 and 4.

A large proportion of such enzymic phosphoryl transfer reactions is thought to proceed via a covalent enzyme-phosphoryl intermediate. These constitute the first major group of the phosphoproteins and are considered in the next section. In a subsequent section we will consider the second major group constituted by the serine phosphate residues involved in regulatory reversible phosphorylation.[1]

1.4 Covalent enzymatic intermediates

On discovery of a new covalently phosphorylated enzyme tentative conclusions can be drawn about the nature of the phosphorylated amino acid residue from a study of the stability of the phosphoenzyme at extremes of pH [10]. Phosphomonoesters are generally acid stable and base labile. In contrast phosphoramidates are acid labile and stable to base. Acyl phosphates are readily discerned by their lability at both pH extremes and their sensitivity towards hydroxylamine [1,10]. Clearly isolation of the phosphoamino acid will facilitate further identification, but possible migration of the phosphoryl group during the hydrolysis of the protein may give rise to anomalous results [16]. Another possible route to identify the nature of the phosphorylated amino acid is via ^{31}P-NMR, since different linkages will lead to characteristic differences in the chemical shifts observed in the spectra. In this way serine phosphate residues have been positively identified in casein and phosvitin [17], *E. coli* alkaline phosphatase [18,19] and phosphoglucomutase [20]. The N-1 phosphohistidine residue of the *E. coli* phosphoryl carrier protein HPr has been studied as well [21,22] and very recently observation of regulatory N-3 phosphohistidines on histones was reported [23].

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[1] Proteins with covalently linked or tightly bound coenzymes could be considered phosphoproteins as well, but these are not the subject of this thesis.

Once the nature of the phosphoamino acid is established its catalytic competence has to be demonstrated. This usually requires the demonstration that the rate of partial reactions is comparable to the overall catalytic rate [24,25]. Not in all cases where a phosphoamino acid was identified could this condition be fulfilled as demonstrated clearly by penetrating studies on hexokinase [25]. In Table 1.2 we present a list of all enzymes that have been proposed to have a covalent intermediate based on the above mentioned criteria.

Recently two new techniques have been introduced that are especially useful when no stable intermediate can be isolated. Thiophosphates or phosphates with isotopically labelled oxygen atoms are chiral and can be used to probe the stereochemistry of the phosphoryl transfer event [3,4]. One in-line displacement leads to an inversion of configuration on the phosphorus atom. If one covalent intermediate is involved, retention of configuration should result from the double displacement reaction. Application of this technique has confirmed the competence of some, but not all

Table 1.2

Covalent-phosphorylated-enzyme-intermediates[a] [e]

I. Phosphoserine	Alkaline phosphatases
	Phosphoglucomutase
II. N-3 Phosphohistidine	Succinyl-CoA Synthetase
	Phosphoglyceromutase
	Bisphosphoglycerate Synthase
	Glucose-6-Phosphatase
	Acid Phosphatase
	ATP-citrate Lyase
	PEP-synthase
	Pyruvate-P-Dikinase[b]
	Enzyme I of PEP-hexose-P-transport system
N-1 Phosphohistidine	HPr of PEP-hexose-P-transport system
	Nucleoside Diphosphokinase[c]
III. Carboxyl-Phosphate	Na^+, K^+ - ATP-ase
	Sarc. Ret - ATP-ase
	Acetate kinase[d]
IV. Nucleotidyl-histidine	Galactosyl-transferase

[a] Data obtained from references 1,3,4,10,18,19,20,21,22,26,28,29,30,35.

[b] A histidyl-pyrophosphoryl intermediate has been identified as well (30).

[c] Personal communication - O. Zetterqvist.

[d] Could not be confirmed using stereochemical analysis (3).

[e] Phosphotyrosine has recently been discovered as the product of a viral protein kinase. Also adenylated and uridilated tyrosine residues have been implicated in regulatory mechanisms. Since these moieties are not intermediates but products of enzymatic reactions they are not considered here.

of the intermediates listed in Table 1.2. For instance, the existence of the acetate kinase acyl-phosphate intermediate has been challenged [3], while others [26] have interpreted the same results as being indicative of a triple displacement mechanism.

In the other technique, known as "positional isotope exchange", scrambling of the β,γ bridging $[^{18}O]$ oxygen with the β nonbridging $[^{18}O]$ oxygen is followed by ^{31}P-NMR [27]. Only when transient formation of phosphorylated intermediates takes place does such scrambling occur. Such experiments are usually performed in the presence of substrate analogues, so that "substrate synergism" will assure optimal rates for the partial reaction [24].

From a comparison of the data in Tables 1.1 and 1.2 it appears that most phosphatases and mutases proceed via a covalent phosphoryl intermediate. Phosphodiesterases do not in general seem to follow such a route, whereas the kinases do not show a preference either way. Clearly phosphohistidine is most commonly employed, which is not surprising since imidazole is likely to be the most effective nucleophile at neutral pH's (ref. 1, page 43) and it can also conserve the energy in the "energy rich" phosphoramidate bond which allows transfer without energy loss [1,22]. The sequences of a variety of phosphohistidine containing active site peptide have been determined [29,30] and no obvious homologies seem to exist [30].

Another interesting property of phosphohistidine residues should be pointed out. The phosphoryl group is found more often on the N-3 than on the N-1 atom (Table 1.2). This seems surprising in view of the demonstrations by ^{13}C-NMR [31,32] and ^{15}N-NMR [33] that the tautomeric form of histidine carrying the lone electron pair on the N-1 is strongly favoured. This is considered to be the form in which it is normally present [32]. The top of Figure 1.1 illustrates this point. For N-3 to be able to act as the nucleophile the unusual form with the lone electron pair carried on the N-3 nitrogen will have to be stabilized. This presumably happens as indicated in Figure 1 with a salt linkage stabilising the hydrogen on the N-1 position. Such linkages are known in serine-proteases [34] and may provide for control over the pKa and orientation of the catalytic histidine residue in the active sites of enzymes.

Much of this dissertation is concerned with studies on the N-3 phosphohistidine of *E. coli* succinyl-CoA synthetase. (Chapters 4,5,6 and 7). Preliminary experiments were performed on ATP-citrate lyase (from rat liver, MW = 440,000), but only a mobile regulatory phosphoserine residue was observed and no evidence of the catalytic phosphohistidine could be found (Chapter 12). Preliminary experiments with rabbit muscle phosphoglycerate mutase were also unsuccessful due to the intrinsic phosphatase activity of this enzyme [35].

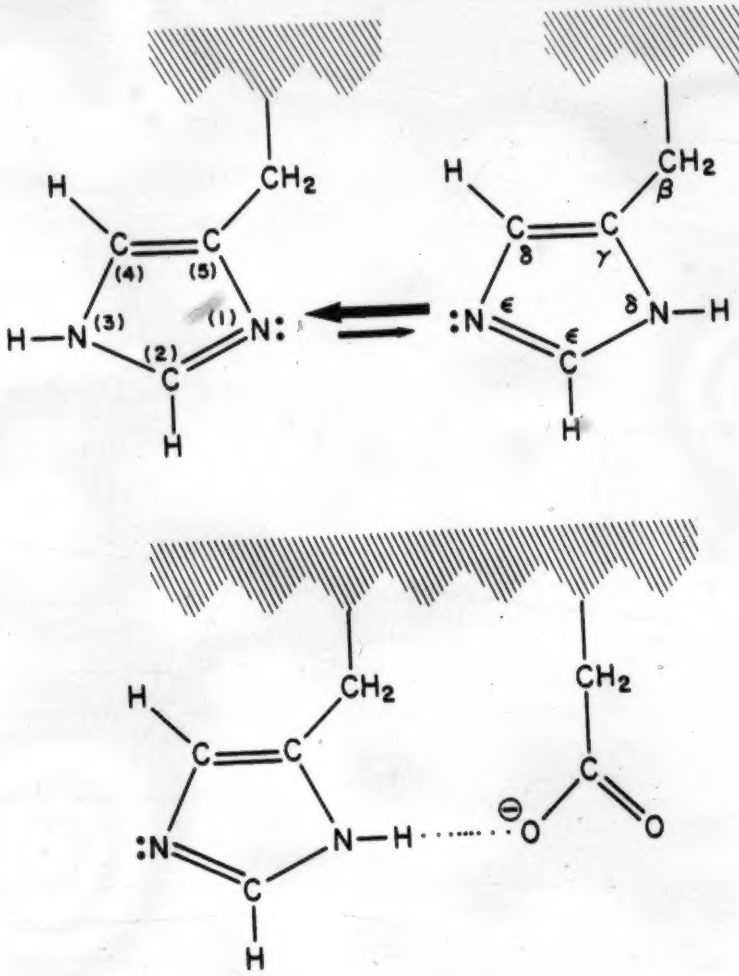

Fig. 1.1 The top of this figure indicates that the predominantly found tautomeric form of deprotonated histidine in peptides and proteins is the one seen in the top left figure carrying the electron pair on the N-1 nitrogen. For N-3 to act as the nucleophile the other tautomer will have to be stabilized in a way as shown in the bottom figure. Also illustrated are the two different nomenclatures that are most commonly found in the biochemical literature. Note that the IUPAC nomenclature numbers the histidine ring the other way around, which reverses the numbering of both carbon and nitrogen atoms. Another nomenclature one can encounter is that were the N-1 is called $N\pi$ and the N-3 is called $N\tau$. In this dissertation we will use the normal biochemical notation as indicated in the top left figure (ref 31,32 and 33).

Table 1.3

Effects of reversible phosphorylation on intermediary metabolism[1]

ANABOLIC	CATABOLIC
(1) Glycolysis-Gluconeogenesis	
(*) Phosphofructokinase ↓	(*) Fructose-di-Phosphatase ↑
(*) Pyruvatekinase ↓	
(+) Pyruvate dehydrogenase ↓	PEP-Carboxykinase ?
(2) Glycogen Synthesis and Breakdown	
(+,o,*) Glycogen synthase ↓	(o) Phosphorylase ↑
(3) Fatty Acid Synthesis and Breakdown	
(+,*) ATP Citrate Lyase ?	(*) Lipase ↑
(+,*) Acetyl-CoA Carboxylase ↓	
(?) Fatty Acid Synthase ↓	
(*) Glycerol-P-Acyl Transferase ↓	
(4) Cholesterol Synthesis and Breakdown	
(+,*) HMG-CoA Reductase ↓	(*) Cholesterol-Ester Hydrolase ↑
(5) Amino Acid Synthesis and Breakdown	
--	(*) Phe, Tyr, Trp hydroxylase ↑

↑ activation; ↓ inactivation; ? unknown; * = phosphorylated by cAMP-dependent protein kinase; o = phosphorylase kinase (Ca^{2+}, cAMP); + = other kinase.

[1] All data taken from Ref. 36 and 37.

1.5 Regulation of enzyme activity via reversible phosphorylation

A wide variety of enzymes and other proteins can become reversibly phosphorylated on specific serine (occasionally threonine) residues. These residues are remote from the active site of enzymes and their modification usually results in marked changes in the activity of the enzyme [36,37]. Glycogen phosphorylase, for example, becomes strongly activated upon modification of its Serine-14 residue [38]. This area of covalent control has received considerable attention in the last decade and the overview presented in Table 1.3 illustrates how wide spread such regulatory sites are. The activities of the protein kinases and phosphatases responsible for the covalent modifications are under hormonal control. Clearly activation of the protein kinases leads to inhibition of all major anabolic processes and an activation of catabolic processes (see Table 1.3). Likewise activation of the protein phosphatases results in the opposite response. Although such global effects on metabolism are now quite well understood, little is known about the mechanism by which these phosphorylations bring about the changes in activity. X-ray crystallographic studies have indicated that Serine phosphate 14 of phosphorylase *b* is in a salt linkage with Arg69 as well as Arg43 of the other subunit and thus interacts with an α helix running through the enzyme to the AMP activator site [38].

Analysis of the protein surface topography indicated the presence of a ring of positive charges around the SerP residue. These are presumably involved in the protein-protein-interaction between phosphatase and its substrate phosphorylase [39]. Subsequent binding of the inhibitor glucose to the active site seems to expel the SerP from the surface of the protein so that it becomes available to the phosphatase [40]. Although these results imply that large domains on the surface of proteins do interact, studies with small peptides have shown that these can serve as reasonable substrates for both the protein kinase [41] and the protein phosphatase [42], albeit at lower Km but similar Vmax. Such studies combined with sequence data [36] have pinpointed the high degee of amino acid sequence homology for such regulatory sites. In addition to phosphorylation of key regulatory enzymes of intermediary metabolism, some of the proteins involved in muscle contraction undergo a similar phosphorylation [43], although the role of such modification is not well understood at this moment. Examples of this are tropomyosin [44], the myosin light chains and multiple sites within the troponin system [43]. ^{31}P-NMR studies of some serine phosphate sites of these last two molecules have been reported [45,46].

In this dissertation we report detailed studies of the serine phosphate sites of phosphorylase *a* (Chapter 4, 8) and tropomyosin (Chapter 8). Also a serine phosphate resonance was observed for ATP citrate lyase (Chapter 12), but due to the instability of this enzyme and the long accumulation times required (50 hours) to obtain a spectrum with reasonable signal to noise ratio we did not pursue this project. Moreover, in Chapter 10 characterisation of a possible regulatory phosphate on bacterial F-pili is reported [47].

Although probably not true members of this group, we will also consider here the serine phosphates of the major egg white protein ovalbumin and the major egg yolk protein phosvitin. It is likely that the phosphorylation of these molecules does not serve a regulatory function but the phosphoserines may be involved in metal binding [16] or they may serve as a phosphorus storage in eggs. Studies on ovalbumin are reported in Chapter 9. The properties of phosvitin are explored in Chapter 11. Preliminary experiments with the milk protein casein seemed promising but heterogeneity of the preparations made unequivocal interpretations of the results impossible.

1.6 Experimental techniques

For the majority of the studies reported in this dissertation we used ^{31}P-NMR. Here we will discuss a few characteristics of this technique that are pertinent to these studies. ^{31}P-NMR has the advantage that the nucleus with spin quantum number 1/2 is 100% abundant so no expensive enrichment procedures need to be employed. This has another advantage in that it can be considered a good reporter group which is only "reporting the news and not making it" like some groups will do that have to be introduced by modification of the protein. Since normally only a few phosphorus-containing compounds are present assignments can usually be readily made. However, the low sensitivity and the long spin lattice relaxation times (T_1) force the experimentalist to use large amounts of proteins, and long accumulation times are necessary to obtain spectra with reasonable signal to noise ratio [48].

The chemical shifts measured in the ^{31}P-NMR spectra are related to shielding by the electron cloud around the P-nucleus. Thus, factors that influence the distribution of electrons are expected to lead to changes in chemical shift. As was pointed out by Van Wazer and Letscher [6] changes only in charge should not necessarily lead to changes in chemical shifts. The shift is more determined by other properties of the phosphorus atom, the bond geometry, the electronegativity of the substituents and the relative amounts of π-bonding. Since change of charge can be accompanied by change in any of these three parameters, differences in chemical shifts are usually observed. Moreover, these three parameters are interdependent and their interrelationships are unfortunately not delineated. As will be seen throughout this thesis the interpretation of such shifts for those reasons remains largely empirical.

More recently, the importance of the effect of the bond geometry on the chemical shifts was demonstrated experimentally [49]. Other factors that may contribute to the chemical shifts of protein bound residues are, for instance, ring current shifts caused by binding of the phosphoryl moiety in the vicinity of an aromatic residue. Also hydrogen bonding may cause small changes in chemical shift. Phosphate binding sites in proteins are not only provided for by arginine residues [7] and metal ions [5,8] but are often found at the N-termini of helices. The recent demonstration that this is caused by an interaction betwen the α helix dipole and the charged phosphate [50] raises the question as to what effect such active site dipole fields may have on the chemical shifts measured in ^{31}P-NMR spectra.

In contrast to the uncertainties about the interpretation of the chemical shifts (for more details see ref. 48 and Chapter 3), we feel that the linewidth and pH titration behaviour of phosphoryl compounds can be analysed with less ambiguity (see Chapter 4 and 8 respectively).

Although ^{31}P-NMR has recently become a popular tool to study metabolism *in vivo* [51,52] and the binding of phosphorylated ligands to proteins [48] it was pointed out in a recent mini review [53] that "....... the use of NMR to study phosphate in proteins is a very underexploited area, particularly considering the importance given to protein phosphorylation as a control mechanism and the utility phosphorus NMR could have in analysing it ......". Also, in an attempt to characterise the linewidth of the ^{31}P-NMR resonance observed for the pyridoxal phosphate residue of serine dehydratase, Schnackerz *et al*. [54] pointed out that "......the predominant relaxation mechanism governing the ^{31}P line width are expected to be dipole-dipole with the neighbouring protons and a contribution from chemical shift anisotropy ......". In contrast Fujitaki *et al*. [23] studying phosphohistidine residues of histones stated that "......the major relaxation mechanism involved here is most likely dipole-dipole with protons of neighbouring amino acid residues ......". It should be clear from these three statements that the study by ^{31}P-NMR of phosphoproteins is still relatively unexploited. Moreover no unanimity exists towards interpretations of chemical shifts (see previous sections) and linewidths (see last two statements). One major aim of this dissertation is to shed light on these kind of questions and to see how far we can carry the interpretations of chemical shifts, linewidths and pH titration behaviour in order to see what biologically relevant information can be obtained by using this approach. The ^{31}P-NMR technique has already shown its utility in delineating stereochemical questions. This was reviewed recently [55] and will not be considered here.

Another experimental approach applied here, which needs further introduction, is the use of the recently developed photo-laser Chemically Induced Dynamic Nuclear Polarization (CIDNP) ^{1}H-NMR technique. Here a flavin dye, excited to its triplet state by brief laser radiation, reacts reversibly with any histidine, tyrosine or tryptophan residues that are accessible to the dye. This leads to transient radical formation, causing nuclear polarization in the side chains of these residues, resulting in large positive or negative enhancements for some resonances in the ^{1}H-NMR spectrum. These enhancements are most readily studied by subtracting a normal spectrum (dark) from one obtained after irradiation with the laser (light) [56-59]. Thus, like chemical modification and photo-oxidation experiments [60] it allows for measuring surface exposure of aromatic amino acid residues. For accessible histidine and tyrosine residues it also allows for an estimate of the amount of H-bonding [59]. Changes in H-bonding and exposure caused either by conformational changes, or aggregation, or the binding of ligands can be followed. In the course of our CIDNP studies on phosvitin (Chapter 11) we learned that quantitative application of the technique was still in its infancy. To help delineate the proper experimental conditions for these and future experiments we studied a few defined "model systems". Since these studies are not directly related to the topic phosphoproteins, they are not part of this thesis. They will be reported elsewhere and only some of the results are taken up in Appendix 2.

1.7 Organization of this thesis

Since a wide variety of biological molecules has been studied here, it seemed more appropriate to organise the material in the form of individual papers which can be read individually rather than trying to present it in the usual way with one general introduction chapter governing the whole background for the dissertation. Therefore each chapter contains its own summary, introduction, results, discussion and conclusions sections and bibliography. This introductory chapter was an attempt to give an overview of the wide area of "phosphoproteins". In the final conclusions chapter we will try to relate our findings discussed in the individual chapters in the broader context of the area of phosphoproteins as outlined here.

The chapters have been put in order such that the first two are not dealing with phosphoproteins but with ^{31}P-NMR studies of small molecules. These two chapters (2, 3) as well as part of Chapter 8 give background information for the following chapters. Chapters 4-12 all deal with several aspects of phosphoproteins studied mainly by ^{31}P-NMR, but other techniques have been employed as well.

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CHAPTER 2

^{31}P-NMR STUDIES OF A POSSIBLE FUTILE CYCLE IN *E. coli*

Summary

The enzyme PEP-synthase catalyses the interconversion from pyruvate to PEP in pyruvate- or lactate-grown *E. coli* cells. Pyruvate kinase is known to catalyse the opposite reaction. Thus if both enzymes are present in the cell at the same time the possibility of a futile cycle arises. This notion is especially credible in view of the fact that no allosteric effectors for PEP-synthase are known at present. Here we report some studies addressing this problem. Three different approaches were used. First we have determined, by means of ^{31}P-NMR spectroscopy, the products resulting from addition of ATP and pyruvate to whole cell extracts. The results showed that futile cycling may indeed occur in lactate-grown *E. coli* but not in glucose-grown cells. This different behaviour could be related to a difference in induction of PEP-synthase upon growth on these two carbon sources. This seems to be a first level of regulation to prevent wasteful use of ATP. Secondly, we could show by means of ^{31}P-NMR studies of reconstituted systems with purified enzyme preparations that ADP inhibits PEP synthase. Thus ADP inhibits futile cycling since it is also the substrate for pyruvate kinase. Thirdly, we measured

induction of the enzyme upon growth on various substrates. This leads us to propose that the induction is regulated at the level of transcription by catabolite repression. Enzyme levels also increased in the stationary phase. Since the routine assay that we used throughout this research was unreliable for whole cell extracts our observations are preliminary and warrant further confirmation.

Introduction

The metabolite pyruvate is at a junction of various metabolic pathways. When a bacterial cell has ample supply of glucose, glycolysis will be active and the majority of the pyruvate will be converted by the pyruvate dehydrogenase complex to acetyl-CoA which is the fuel for the tricarboxylic acid cycle and fatty acid synthesis. When the glucose supply is low the cells can shift to gluconeogenesis. Glycolysis and gluconeogenesis share the majority of the enzymes of the glycolytic pathway. However the conversion of PEP to pyruvate via pyruvate kinase is virtually unidirectional. Thus gluconeogenesis from pyruvate (or lactate or alanine) is a more complex process. Eukaryotes can bypass this via the sequential actions of the mitochondrial anaplerotic enzyme pyruvate carboxylase [1] and the gluconeogenic enzyme PEP-carboxykinase. This last enzyme is responsible for gluconeogenesis not only from pyruvate, but also from succinate or other TCA-cycle intermediates [2]. Some prokaryotes, including *E. coli*, do not have this option, however. Although they possess an inducible PEP-carboxykinase [3,4,5] they lack the enzyme pyruvate carboxylase [6,7], making use of PEP-carboxylase to replenish the TCA cycle intermediates that have been used for biosynthesis [6,7,8]. Unfortunately, this enzyme is of no help to assure gluconeogenesis or replenish TCA-cycle intermediates in pyruvate grown *E. coli*. However, the organism grows readily on this carbon source. This is made possible by induction of the enzyme PEP-synthase [9,10]. The synthesis of this enzyme is repressed when glucose is the carbon source [10]. PEP-synthase catalyses virtually unidirectionally the syntheis of PEP from pyruvate [10,11] and is in that respect antagonistic to pyruvate kinase. Thus the simultaneous presence of both of these enzymes could give rise to a futile cycle. Usually the presence of allosteric effectors prevents such wasteful use of ATP. For example, phosphofructokinase and fructose-diphosphatase can be effected in opposite ways by the same allosteric effectors [12]. The activity of homogenous preparations of PEP-synthase was not effected by addition of a wide variety of metabolites [13,14], although studies with impure preparations had been suggestive in this respect [15]. If the activity of PEP-synthase is not under allosteric control, the possibility of a futile cycle seems far from remote. In the presence of excess adenylate kinase [16], the following scheme would apply:

$$\begin{array}{lll} \text{ATP + pyruvate + } H_2O & \rightarrow \text{PEP + AMP + Pi} & \text{(PEP-synthase)} \\ \text{ADP + PEP} & \rightarrow \text{ATP + pyruvate} & \text{(pyruvate kinase)} \\ \text{ATP + AMP} & \leftrightharpoons \text{2ADP} & \text{(adenylate kinase)} \\ \hline \text{ATP + } H_2O & \rightarrow \text{ADP + Pi} & \end{array}$$

E. coli has two different pyruvate kinase enzymes. The first one is constitutive and is allosterically activated by AMP or ribose-5P [17-21]. Product inhibition of PEP-synthase by AMP and allosteric activation of the constitutive pyruvate kinase by this nucleotide could be sufficient to prevent futile cycling under these conditions [14]. The second form of pyruvate kinase is induced by growth on glucose. This enzyme is activated, like the eukaryote enzyme, by fructose 1,6 diphosphate [17,18,21-23]. One would predict futile cycling to occur especially after growth on pyruvate with a subsequent carbon source upshift to glucose.

Here we describe preliminary ^{31}P-NMR measurements performed on whole cell extracts of *E. coli* B grown on lactate or glucose. The usual assays for PEP-synthase [23] or pyruvate kinase [24] measure only the decrease or increase in the pyruvate concentration. All other metabolites involved are phosphorylated and since they are all compounds with differently substituted phosphoryl moieties they are well resolved in a ^{31}P-NMR spectrum. This approach will allow us to investigate the possibility of a futile cycle by following the increase and decrease of the substrates and products involved. Studies can be done in the absence or presence of known activators, inhibitors or nonhydrolysable analogues. This experimental approach is not just limited to the study of whole cell extracts. The results can be complemented by reconstitution experiments using purified enzyme preparations. In addition to these spectroscopic observations we have also studied the induction of PEP-synthase under a variety of growth conditions.

Experimental procedures

PEP-synthase was purified in our laboratory by Ms. S. Narindrasorasak and it was stored as described elsewhere [13,14]. Pyruvate kinase and adenylate kinase were both obtained from Sigma Chemical Company and were used without further purification. All other chemicals and nucleotides were analytical grade. Total protein concentrations were determined by the Biorad protein assay. PEP-synthase was assayed by monitoring the ATP dependent removal of pyruvate at 37°C [24].

E. coli B was grown in a minimal phosphate medium with a carbon source concentration of 0.5% (w/v). The cells were collected by centrifugation at 5000 rpm for 15 min at 4°C. The supernatant was discarded and the cells were resuspended to a constant cell density. (E.g. 1 liter of glucose-grown cells, with a final density of the culture of OD 600nm = 3.0, was resuspended in 30 ml). Previous trials had shown that a higher density of cells was not disrupted efficiently by the French press (3 cycles at 16000 psi, 4°C). These extracts were centrifuged for 30 min at 12,000 rpm (4°C) and were then used without further purification. Activity and ^{31}P-NMR measurements were always performed within a few hours of the preparation of the extract to minimize the decrease in PEP-synthase activity [24]. NMR samples (1.5 ml total volume) were prepared by mixing 1 ml of extract with stock solutions of ATP, pyruvate, $MgCl_2$ and KCl dissolved in a Tris buffer in D_2O (pH 7.0). The final concentrations were Tris 50 mM and all other reagents 25 mM. All NMR experiments were performed at 28°C. All other conditions used to acquire the spectra were identical to those described in Chapter 10, except that the spectra were not proton decoupled.

Results

Reconstitution experiments. Some of the spectra that were obtained by using purified enzymes are shown in Figure 1. For example, Fig. 1a shows the spectrum of 25 mM ATP in the presence of a catalytic amount (1-2 units) of homologous PEP-synthase. The enzyme reaction was started by addition of pyruvate and the progress of the reaction was monitored by obtaining spectra at different time intervals. Fig. 1b shows such a spectrum obtained after 20 min. of incubation. As expected the levels of ATP decrease and concommitantly the resonances observed for products AMP, Pi and PEP increased. When this experiment was repeated in the presence of excess

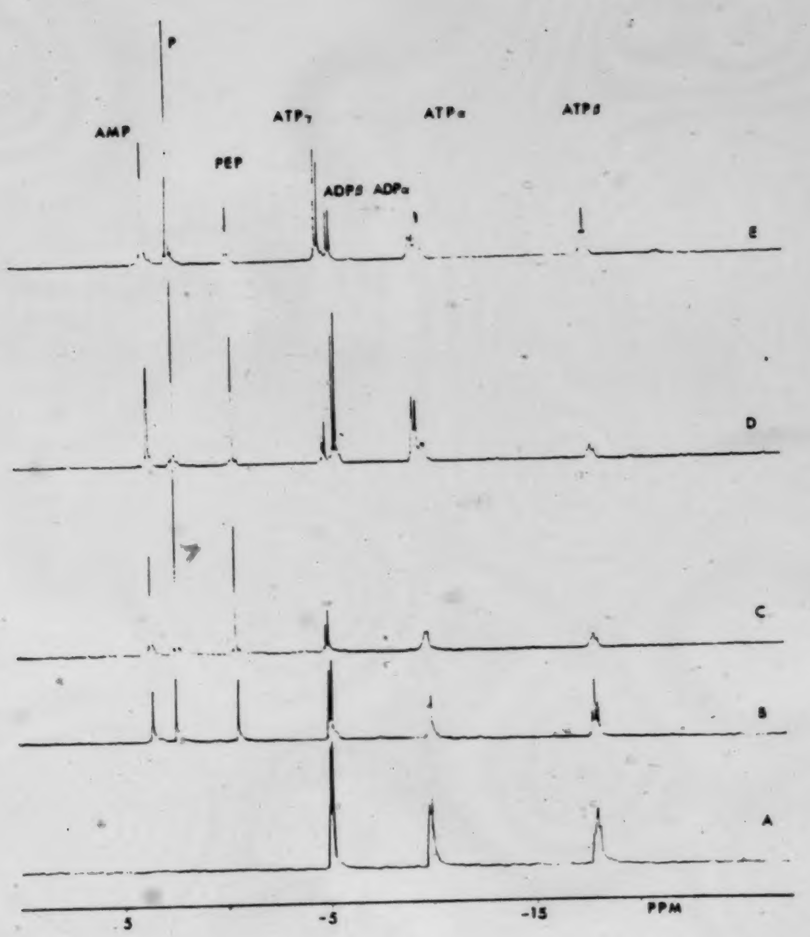

Fig. 2.1 ^{31}P-NMR spectra of reconstitution experiments using purified PEP-synthase and pyruvate kinase. All spectra represent 50 scans and are plotted with 1 Hz additional linebroadening caused by the computer digital filtering. (a) 25 m ATP, and PEP-synthase; (b) same as previous, 20 min after addition of pyruvate; (c) same as previous, 2 hours after addition of pyruvate; (d) same as previous after addition of ADP; (e) same as previous, 36 min after addition of pyruvate kinase.

adenylate kinase (10-15 units) only a small amount of AMP accumulated over the same time course. Obviously it was directly transformed to ADP as evidenced by the appearance and increase in the ADP α and β resonances in the spectra (data not shown). Most noteworthy was that the progress curves of these two reactions indicated that less PEP was formed over the same time period if adenylate kinase was present. This unexpected observation can only be explained by assuming that ADP is a more effective inhibitor of the enzyme than product inhibition by AMP. Experiments with partially purified enzyme have confirmed that ADP inhibits the enzyme [15]. Unfortunately no such studies have been reported yet for purified preparations of the enzyme [13,14]. Fig. 1c shows the same mixture of Fig. 1b after two hours of exposure. Apparently equilibrium was reached since this spectrum did not change over a 30 minute time period. It is obvious from this spectrum that the reaction towards PEP formation is strongly favoured. This result is in agreement with experiments by others [10,11]. In Figure 1d we have simulated the action of adenylate kinase by addition of ADP to the equilibrium mixture of Fig. 1c. This was necessary in order to be able to study the effect of addition of pyruvate kinase (1-2 units) in the absence of adenylate kinase. Again we followed the progress of the reaction by obtaining NMR spectra at different time intervals. The spectrum obtained after 36 min is shown in Fig. 1e. As expected, the levels of ADP and PEP have decreased and the amount of ATP has increased. Note that the AMP and Pi peak have also increased slightly indicating that PEP-synthase must have utilised some of the newly formed ATP. Thus futile cycling between PEP-synthase and pyruvate kinase has occurred. The increment in AMP and Pi was small however given the time course of this experiment. This is in agreement with the concept that ADP and AMP inhibit PEP-synthase and thus block futile cycling. Further studies employing reconstituted mixtures confirmed the results as shown in Fig. 1 and they are consistent with the idea of futile cycling which could be slowed down considerably by ADP inhibition of PEP-synthase.

Experiments with whole cell extracts. Cultures of *E. coli* B were grown with either lactate or glucose as the carbon source. They were harvested in late log phase (OD 600nm $\approx$ 1.5). ^{31}P-NMR spectra were obtained on whole cell extracts after addition of ATP and $MgCl_2$. When no drastic changes occurred in the spectra pyruvate was added and a series of spectra was collected. One such series is shown in Fig. 2 for lactate-grown *E. coli*. It shows that the levels of ATP go down, that ADP goes through a maximum and that the final result is the formation of AMP and Pi. This behaviour is in support of a possible futile cycle that can occur in these cells. Very similar results were obtained with other extracts prepared from log-phase lactate-grown *E. coli*. Only the time course of these experiments varied between samples.

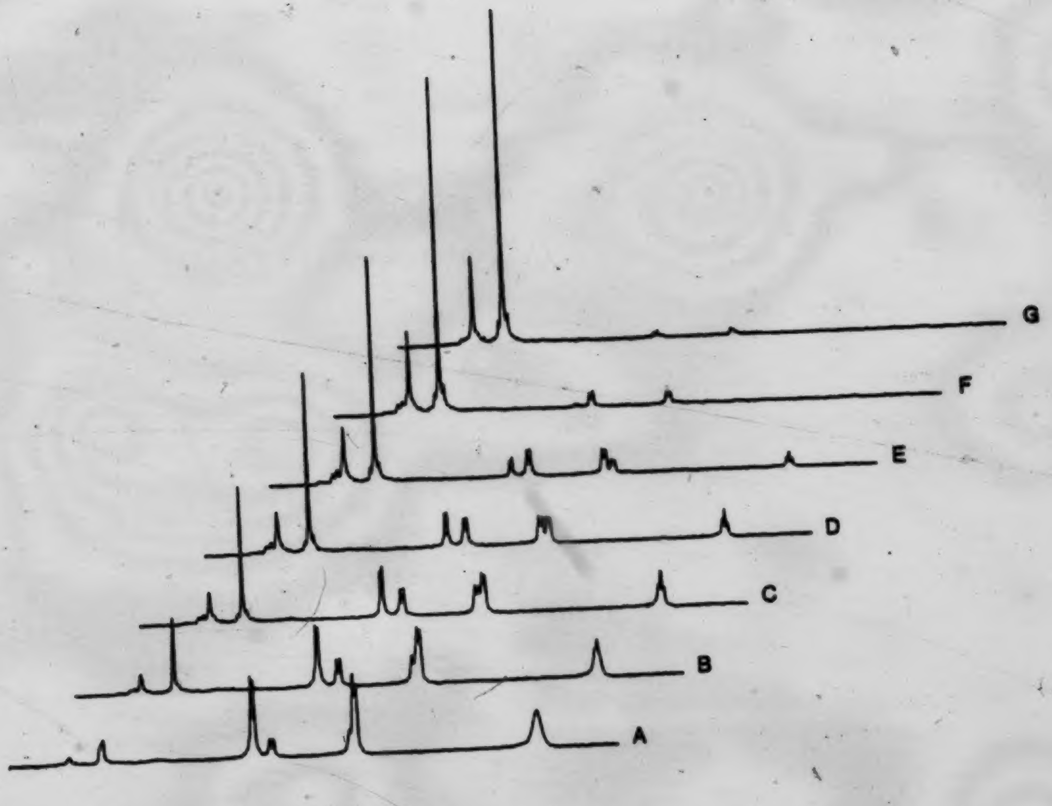

Fig. 2.2 ^{31}P-NMR spectra of ATP and pyruvate added to a whole cell extract of lactate-grown *E. coli* B. For the assignment of the peaks see Fig. 1. In spectrum 2a these are from left to right AMP, Pi, ATPγ, ADPβ, ADPα, ATPα and ATPβ. Conditions were 250 scans, LB = 1 Hz. (a) after 4 min; (b) 18 min; (c) 36 min; (d) 52 min; (e) 68 min; (f) 124 min.

Since these samples were prepared at a constant cell density and not at a constant activity of PEP-synthase such differences are not unexpected. Results with dialysed extracts were also quite similar. Such samples had lost some of their PEP-synthase activity upon dialysis due to the instability of the enzyme in crude extracts [24].

A comparison of Fig. 1 and Fig. 2 shows that no resonance is observed for the PEP in the whole cell extracts. This must be the result of other enzymic activities in the extracts that remove this metabolite. These will be considered in turn in the following:

- It is unlikely that PEP-carboxylase is active since this enzyme requires FDP for activity [8].
- These preparations are thought to be devoid of membrane material and thus also in PEP dependent sugar phosphotransferase activities [26].
- The activity of alkaline phosphatase is expected to be negligible since this enzyme is induced only upon growth on low phosphate media [27].
- Since no build up of glycolytic intermediates is observed in the ^{31}P-NMR spectra possible gluconeogenesis from PEP seems unlikely.

For these reasons we feel that the majority of the PEP has been utilised by pyruvate kinase. This would support our idea of futile cycling.

In contrast to our results with the lactate-grown cells, log phase cultures grown on glucose, extracted at an equal density of cells, did not show similar behaviour. Instead after 10 hours roughly half of the original amount of ATP was still detectable in these spectra; the only resonances that had appeared in the spectra were assigned to ADP and Pi (data not shown).[1] Thus no futile cycling occurs in log phase glucose-grown cells presumably since PEP-synthase is not induced (see next section). It should be mentioned here that some of these glucose-grown samples had apparently accumulated paramagnetic metal ions. Their presence resulted in a broadening of the resonances in the ^{31}P-NMR spectra.

Induction of PEP-synthase. The induction of this enzyme was studied by assaying for PEP-synthase activity on samples grown under a wide variety of conditions. Normally two identical samples were grown and these were each assayed in duplicate. In the course of these experiments we encountered several problems with the assay. In the first place the extracts contain some pyruvate and ATP. Secondly, they may contain enzymatic activities that affect the pyruvate or PEP concentration. For example, the pyruvate dehydrogenase complex may utilize some pyruvate [28]. Another possibility is futile cycling with pyruvate kinase

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[1] This observation strongly suggests that the ATP and ADP hydrolysis observed in the lactate grown cells (see Fig. 2) is not caused by nonspecific hydrolytic- or phosphatase activities.

(see previous section) which regenerates pyruvate from PEP resulting in an underestimate of the activity. A third problem we noticed was that the activity of purified enzyme preparations varied over a day. This was likely caused by aging of some of our stock solutions. Given these uncertainties we will not present quantitative specific activity (units/mg total protein) data but rather present only the preliminary trends that could be discerned from our results.

- PEP-synthase was always induced in cultures that had reached stationary phase. This result was independent of the carbon source used for growth.
- Growth on C3 sources like pyruvate, lactate or alanine resulted in induction in the log phase. Moreover growth on glycerol, succinate and acetate had the same effect, whereas the presence of glucose (and fructose) repressed synthesis in the log-phase.
- In glucose-grown cultures induction takes place only after the stationary phase is reached.
- In lactate-grown cultures the specific activity remains relatively level throughout the whole growth curve.
- Addition of α-methylglucoside to lactate-grown cultures at various growth stages did not result in any change in the induction of the enzyme.

Discussion

The results of the reconstitution experiments (Fig. 1) and the data obtained with whole cell extracts (Fig. 2) are

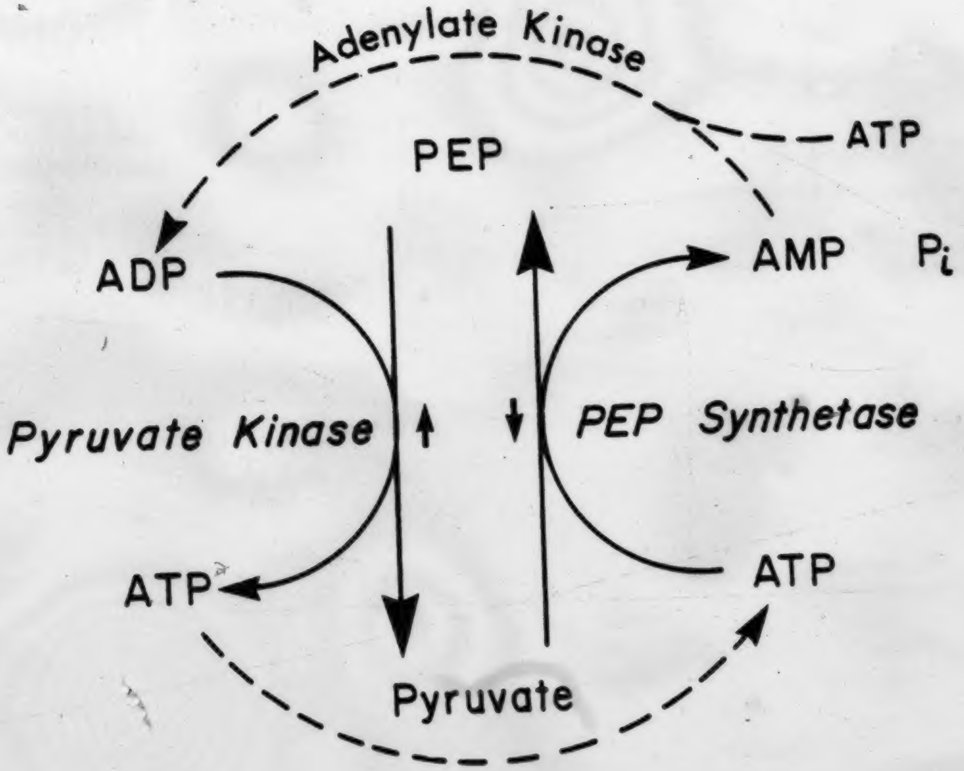
Adenylate Kinase
ATP
PEP
ADP
AMP
P_i
Pyruvate Kinase
PEP Synthetase
ATP
ATP
Pyruvate

Fig. 2.3 Schematic representation of the futile cycle as it may occur in lactate-grown *E. coli* B.

in agreement with our hypothesis that futile cycling may occur in lactate-grown *E. coli*. This result is summarized in Fig. 3. It can be seen that adenylate kinase plays a governing role in this process. Our results with the whole cell extracts (Fig. 2) seem to support the idea that the levels of this enzyme in *E. coli* are sufficiently high that the reaction is close to equilibrium *in vivo* [16]. Different levels of control exist in the bacteria to prevent the futile cycling. These are considered in turn in the following.

(1) It is of interest that no wasteful use of ATP occurs in glucose-grown cells, but only in lactate-grown cells. This must be due to the repressive effect of glucose on the induction of PEP-synthase and represents the first level of regulation available to the bacterium. Although the enzyme is strictly necessary for growth on only pyruvate (and related carbon sources) we have shown that it is also induced by log phase growth on several other carbon sources. Apparently no specific pyruvate induction mechanism exists. Likewise the metabolically related enzyme PEP-carboxykinase is not specifically induced by growth on succinate [4] but is controlled by catabolite repression and inducer exclusion [26,29]. These effects can usually be counteracted by addition of cyclic AMP to glucose-grown bacteria [29,30]. Thus in the case of PEP-carboxykinase addition of cyclic AMP to glucose grown log phase cultures resulted in induction

[4]. Recent experiments have shown that active uptake of glucose via the PEP-driven sugar phosphotransferase system [26,30,31] results in a decrease in the activity of the membranous adenylate cyclase activity which in turn results in a drop in the intracellular cyclic-AMP levels. We reasoned that addition of the nonmetabolisable glucose analogue α-methylglucoside, which is transported in the same way as is glucose, would result in a decrease in the cyclic AMP levels in lactate-grown cultures and hence would lead to a low level of induction of PEP-synthase. Our experiments unfortunately showed that levels of the enzyme did not change. Since we did not perform α-methylglucoside uptake control experiments we cannot be sure that the analogue was transported into the cell. The final answer will have to await new experiments with cyclic-AMP added to glucose-grown cultures.

It is noteworthy that our result on the induction of PEP-synthase are in close agreement with those obtained for the enzyme pyruvate-P-dikinase of *Acetobacter xylinum* [32]. This enzyme is very similar in function and mechanism to PEP-synthase and is found in plants and certain bacteria [14].

We showed that PEP-synthase activity was always induced in the stationary phase. Other enzyme activities are subject to a similar control. These are PEP-carboxykinase [4], glycogen synthesis [33,34] and proteolysis [35]. The levels of the regulatory nucleotides cyclic AMP [31] and ppGpp [33] both peak at the onset of the stationary phase. This increase in ppGpp was correlated to the increase of glycogen synthesis [33,34] and proteolysis [35]. However the induction of PEP-carboxykinase in stationary phase could not be explained by an increase in the levels of either of the nucleotides [4]. It would be of interest to determine whether induction of PEP-synthase in the stationary phase is regulated in analogous fashion to that of PEP-carboxykinase.

(2) Another means of regulation is at the level of allosteric control. The activities of the two different forms of pyruvate kinase are reported to be controlled by different metabolites [17-23]. For PEP-synthase no controls have been identified [14]. Here we have found indications that the presence of ADP results in a decrease of the activity. This effect deserves further study since it may be important in limiting futile cycling. Also, allosteric controls seem to be absent for *E. coli* PEP-carboxykinase [36,37].

(3) Other more subtle control mechanisms may exist. Recently metal ions have been implicated in the regulation of the activity of several enzymes related to pyruvate metabolism in *E. coli* [37-39]. It may be of interest that we have observed accumulation of paramagnetic metal ions in glucose-grown cells and not in lactate-grown cells. Little is known about the relative intracellular concentrations of

metal ions.

(4) The potential for control also exists at the level of availability of substrates and the build up of products. Thus AMP inhibition of PEP-synthase may play a role [14], but the activity of adenylate kinase in adequately energised cells will decrease its level to negligible amounts. Likewise the other product PEP will be converted into glycolytic pathway intermediates or consumed by PEP-carboxylase. The importance of yet another system was pinpointed by studies with mutants totally devoid of pyruvate kinase activity [40]. These only grow well on sugars that are substrates for the PTS system. Thus the concerted transport and phosphorylation of such sugars by PEP via the PTS system apparently results in sufficient production of pyruvate to assure normal growth. We have shown here that the synthesis of PEP-synthase is repressed by growth on PTS substrates. Thus the enzyme cannot be responsible for reconverting pyruvate to PEP to aid in the transport of these PTS sugars. Since PEP synthase is induced in stationary phase it may however be instrumental for a "kickstart" of the transport of PTS sugars when the cells are transferred to fresh media.[2]

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[2] Yet another system exists in *E. coli* that is involved in the interconversion from PEP to pyruvate. It requires the sequential actions of the enzymes PEP-carboxylase [8], malate dehydrogenase [41] and the NAD^+ or $NADP^+$ specific malic enzymes [38,39]. Alternatively the route via malate dehydrogenase, malic enzymes and PEP-synthase provides another route for gluconeogenesis from succinate in addition to the normal route via PEP-carboxykinase. These are not considered further here.

It is clear from the foregoing that the required regulation can occur at several levels in interplay and that we do not yet understand all of the intricacies. Although we have shown here that futile cycling is feasible in whole cell extracts it remains to be demonstrated *in vivo*. In this respect it should be mentioned that futile cycling between phosphofructokinase and fructose diphosphatase apparently does not occur in *E. coli* [42].

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CHAPTER 3

^{31}P-NMR STUDIES OF THE METHYLENE- AND FLUORO-ANALOGUES OF ADENINE NUCLEOTIDES; EFFECTS OF pH AND Mg^{2+} BINDING

Summary

The ^{31}P-NMR spectra of methylene- and fluoro-analogues of adenine nucleotides have been studied as a function of pH and Mg^{2+} concentration. Both structural modifications lead to characteristic changes only in the chemical shifts for the substituted phosphorus resonances. As expected, the spectra of the fluoro-analogues are not a function of pH. Mg^{2+} titrations show that both fluoro-analogues bind the metal ion less strongly than ATP or ADP, respectively. For the analogues AMPPCP(β,γ) and AMPCP(α,β), a pKa near 8.1 was deduced for the titration of the terminal phosphoryl group; in the presence of Mg^{2+} this value is lowered to about 6.3. The other ATP analogue AMPCPP(α,β) has a titration behaviour virtually identical to that of ATP. Therefore, at physiological pH all adenine nucleotides will be fully deprotonated in the presence of Mg^{2+} ions. A comparison of the magnitudes and directions of the chemical shift changes observed due to protonation leads to the conclusion that these are caused by changes in the structure of the polyphosphate chain. Changes observed upon Mg^{2+} binding are similarly explained. Mutual titrations indicate that both methylene ATP analogues can bind one Mg^{2+} tightly and a

second ion more weakly. The methylene analogue of ADP binds only one Mg^{2+}. These data show that the methylene analogues compare favourably with ATP and ADP with respect to metal binding properties. We believe that the greater sensitivity of the chemical shifts to protonation and Mg^{2+} binding shown here may be a useful property in future ^{31}P-NMR studies of enzyme-nucleotide interactions. A comparison of our results with those reported for imido- and thio-adenine analogues suggests that the ^{31}P-NMR spectra for metal complexed nucleotides free in solution are probably the fast-exchange average of all metal-coordination structures possible. This raises questions about the validity of direct comparisons of these spectra to those observed for enzyme-bound nucleotides.

Introduction

ATP and ADP exist under physiological conditions predominantly as 1:1 Mg^{2+} complexes. It is not surprising that one of the first biochemical applications of ^{31}P-NMR concerned the changes observed in the three line spectrum of ATP induced by additions of metal ions (Cohn and Hughes, 1962). The authors concluded that ATP Mg^{2-} exists predominately as the β,γ bidentate complex in solution. Several groups have subsequently studied the same system performing similar experiments; unfortunately, no unique structure has emerged from these efforts (Brown *et al.*, 1973; Tanswell *et al.*, 1975; Tran-Dinh *et al.*, 1975; Tran-Dinh and Roux, 1977; Ramirez and Mariceck, 1980; Bishop *et al*., 1981). One of the approaches most commonly used is to measure changes in the chemical shifts induced by additions of diamagnetic metal ions. Recent ^{31}P-NMR studies on the thiophosphate analogues of adenine nucleotides led Jaffe and Cohn (1978) to conclude that this last method cannot be used to determine the absolute coordination structure of metal complexes of nucleotides.

Since virtually all enzymes that use or produce ATP have a requirement for Mg^{2+} ions, the ^{31}P-NMR method has been used recently to study ATP-Mg-enzyme complexes. Again, no unique coordination structures of ATP in the active sites of enzymes have yet been observed. (For review see Cohn and Rao, 1979.) More progress has been made in experiments making use of ATP analogues with substitutions on the polyphosphate chain. In particular, the thiophosphate analogues and the exchange-inert metal-ATP complexes have been useful in probing the metal coordination of enzyme-bound ATP and ADP. Taken together, the results indicate that different enzymes exhibit specificities for different metal-nucleotide complexes (Eckstein, 1979, 1980; Mildvan, 1979; Cleland and Mildvan, 1979; Dunaway-Mariano and Cleland, 1980).

Another class of ATP analogues are those with non-hydrolyzable imido and methylene substitutions for polyphosphate bridge oxygens. The former has been more popular since it more closely resembles the structure of ATP (Yount *et al.*, 1971), but this analogue is somewhat unstable and recently it was shown that commercial preparations could contain contaminating ATP (Penningroth *et al.*, 1980). The methylene analogues are more stable, and unlike the imido analogue, the α,β as well as the β,γ analogue of ATP can be readily synthesized (Yount, 1975). A group of ATP analogues which has not received comparable attention are those in which the terminal titratable -OH group is replaced by a fluorine atom. Since these mimic the protonated nucleotide species in net charge at all pH values, they can be used to probe nucleotide binding sites to determine whether binding of mono- or dianionic phosphates is favoured (Yount, 1975; Withers and Madsen, 1980).

The major thrust of this publication is to compare the pKa's of the terminal phosphate and the Mg^{2+} binding properties, as determined by ^{31}P-NMR, of methylene- and fluorinated ATP and ADP analogues with those reported for ATP, ADP and their respective imido- and thio-analogues.

Experimental Procedures

ATP and ADP were obtained from Terochem Laboratories (Edmonton, Canada) both as the disodium salts. AMPCPP(α,β) and AMPCP(α,β) were purchased from Sigma Chemical Company, as the dilithium salt and the free acid, respectively. AMPPCP(β,γ) was obtained from Boehringer as the tetralithium salt, and ATPγS and ADPβS were obtained from the same source as the tetra- and trilithium salts. AMPPNP was purchased from P.L. Biochemicals as the tetrasodium salt and from Boehringer as the tetralithium salt. ATPγF and ADPβF were a gift from Dr. H. Baer, Department of Pharmacology, University of Alberta, and were originally synthesized in the laboratory of Dr. F. Eckstein, Max Planck Institute, University of Göttingen, Germany. AMPF was a gift from Dr. S. Withers and was synthesized and purified as reported (Withers and Madsen, 1980). The disodium salt of monofluorophosphate was from Alfa. Tris (ultra pure) was obtained from Schwarz-Mann, KCl from Anachem, and $MgCl_2$ and EDTA were Baker-Analyzed. D_2O was obtained from Biorad.

Most nucleotides were judged by ^{31}P-NMR to be at least 98% pure. Exceptions were certain batches of AMPPNP, which especially upon prolonged storage contained contaminants, predominantly $ADPNH_2$ (see Tran-Dinh and Roux, 1977, and Penningroth *et al.*, 1980). ATPγS contained at least 5% ADP and ADPβS had a trace of AMP. The monofluorophosphate contained a 1-2% pyrophosphate. All nucleotides were dissolved in a buffer of 40 mM Tris, 80 mM KCl, 20% D_2O that had been passed through a column of Chelex. In experiments without $MgCl_2$, 0.1 mM EDTA was also included to complex residual paramagnetic impurities. Since Ramirez and Mariceck (1980) have shown that monovalent as well as divalent cations can bind to ATP and ADP, the buffer employed here may have some effect on the chemical shifts and thus also on observed pKa's. Stock solutions of $MgCl_2$ for the Mg^{2+} titrations were made up in the same buffer to obviate changes in the ionic strength during the experiment. No corrections were made in pH measurements for 20% D_2O solutions. pH titrations were performed by adding small aliquots of 1 N or 0.1 N HCl and NaOH solutions. All titrations were performed at nucleotide concentrations below 7.5 mM, where self-association of nucleotides should not occur (Lam and Kotowycz, 1977).

All ^{31}P-NMR spectra were recorded at 109.29 MHz on a Bruker HXS-270 instrument operating in the Fourier-transform mode. The spectrometer is equipped with quadrature phase detection and is interfaced to a Nicolet 293-B pulser and Nicolet-1180 computer. All spectra were obtained with proton-decoupling during acquisition unless otherwise stated in the text. A typical experiment consisted of a 0.4 sec acquisition time, 2 sec recycle time, 60° pulse angle, 5000 Hz sweep width and 4K memory size. These spectra were sufficiently accurate for determination of the chemical shift. When higher resolution was necessary (e.g. to measure coupling constants), this was achieved either by using a larger memory size or by extending the free induction decays artificially by zerofilling. The usual sample size was 1.5 ml in 10 mm Wilmad precision tubes fitted with teflon vortex plugs. All ^{31}P chemical shifts are referenced to external 85% H_3PO_4. Upfield shifts are given a negative sign. The temperature for all NMR experiments was controlled at 27 ±

1°C. ^{19}F NMR spectra were obtained with the same instrument at a frequency of 254 MHz.

Results

A. Assignment of peaks; effect of substitution on the chemical shift. Fig. 1 shows the ^{31}P-NMR spectra of ATP, ADP and their respective methylene- and fluoro-analogues. All spectra were obtained at pH 8.0; as shown later most are in the unprotonated form but AMPPCP(β,γ) and AMPCP(α,β) are near their pKa. It can be readily seen that in all cases the phosphorus resonances that are shifted relative to ATP and ADP are the ones with the substitutions. For example, the β- and γ-phosphorus resonances of the fluoro-ADP and ATP analogues can be unequivocally assigned as the upfield shifted resonances, since they are directly coupled to the fluorine atom which gives rise to a large *JPF* coupling constant. (935 Hz). Thus fluorine substitution leads to an upfield shift of about 12 ppm on the substituted phosphorus atom, whereas the nonsubstituted P resonances are only slightly affected.

For the methylene analogues, where the carbon substitution is in the bridging position, both neighbouring phosphorus atoms are affected and show large downfield shifts between 20 and 30 ppm, but again the unsubstituted phosphorus is hardly affected. Therefore the assignments for the ATP analogues can be readily made, since the β resonance

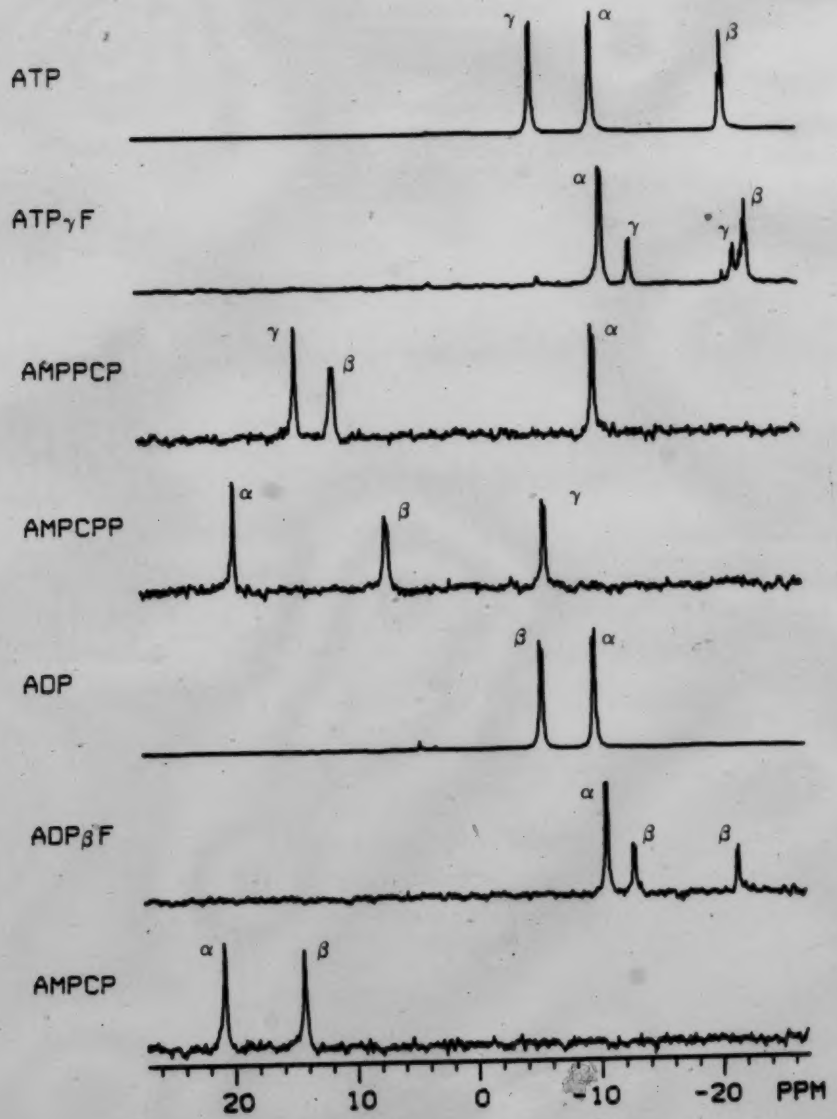

Fig. 3.1 ^{31}P-NMR spectra of ATP, ADP and its methylene and fluoro analogues. Conditions to acquire spectra were described in Materials and Methods. (a) ATP (25 mM, 325 scans); (b) ATPγF (4.5 mM, 700 scans); (c) AMPPCP(β,γ) (1.5 mM, 1000 scans); (d) AMPCPP(α,β) (1.5 mM, 1000 scans); (e) ADP (25 mM, 325 scans); (f) ADPβF (2.0 mM, 700 scans); (g) AMPCP(α,β) (1.5 mM, 1000 scans). All spectra shown are plotted with an artificial linebroadening of 5 Hz. Assignments of the peaks is as indicated in the figure. (See text).

is easily identified due to its triplet or quartet (doublet of doublets) nature. Earlier results obtained with methylene-ATP analogues (Myers, 1967; Granot *et al.*, 1980a, 1980b) showed similar chemical shifts and coupling constants. The only difficult assignment is that for AMPCP(α,β). Since the β-P of ADP shows an identical chemical shift as the γ-P of ATP, we reasoned that the β-P of AMPCP(α,β) should be identical to the γ-P of AMPPCP(β,γ). Similarly, the α-P of AMPCP(α,β) should be close to the α-P of AMPCPP(α,β). We thus assigned the more downfield peak to the α and the other peak to the β phosphorus. Note however that this has inverted the position of the two peaks in the spectrum as compared with ADP, but we are reasonably confident of this assignment since the upfield resonance has a coupling pattern with ^1H, characteristic of the neighbouring $-CH_2-$ resonance and that for the α-P is far more complex due to proton coupling with both the methylene- and ribose ring protons.

In Table 1, we have collected the chemical shift positions of ATP and ADP and all analogues published so far, all at pH values where the terminal phosphate is unprotonated. Direct comparison shows that in all cases studied so far changes in the chemical shift position are induced only by substitution. These changes therefore are most likely associated with the different electronegativities of the substituents on the phosphorus atom and not with changes in

Table 3.1

Chemical shifts of ATP, ADP and their analogues

Compound	Ref[a]	pH	Chemical Shifts (ppm)		
			α	β	γ
ATP	1,4	8.0	-10.9 (d)[b]	-21.3 (t)	-6.0 (d)
AMPPNP (β,γ)	2	11.5	-10.1 (d)	-6.47 (q)	-0.08 (d)
AMPPCP (β,γ)	4	11.0	-10.1 (d)	14.5 (q)	12.4 (d)
AMPCPP (α,β)	4	9.5	19.6 (d)	6.9 (q)	-5.4 (d)
ATPγS	1	8.4	-10.6 (d)	-22.0 (q)	35.0 (d)
ATPγF	4	8.0	-10.1 (d)	-22.6 (t)	-17.5 (sd)
ADP	1,3	8.0	-9.7 (d)	-5.3 (d)	
AMPCP (αβ)	4	11.0	23.0 (d)	12.0 (d)	
ADPβS	1,4	8.0	-11.1 (d)	33.9 (d)	
ADPβF	4	8.0	-10.8 (d)	-17.3 (sd)	

[a] References listed with Table 4.

[b] d = doublet, t = triplet, q = quartet, sd = split doublet due to P-F coupling (935H_Z).

bond angle which are also known to give large changes in the chemical shifts (Gorenstein *et al.*, 1976). For the nonbridging positions we see that the order of chemical shifts is (downfield to upfield) S > O > F. Likewise we see for the bridge position that the order is C > N > O. From their relative positions in the periodic table it is clear that a close corelation exists between electronegativity of the substituent, and the observed chemical shift of the resonance. (i.e., Higher electronegativity leads to upfield shifts.) The data of Table 1 also show that no large changes in the nonsubstituted resonances occur, implying that large differences do not exist between the structures of the polyphosphate chain of these compounds in solution.

B. Effect of pH on fluorine analogues. Both ATPγF and ADPβF were titrated between pH 4 and 10. Within this range no changes were observed in the chemical shifts or coupling constants of any of the observed peaks. When the same samples were studied by ^{19}F-NMR, identical coupling constants were measured and no other fluorine-containing compounds were detected. Similar results were obtained with AMPF (S. Withers, unpublished observations). A comparison of fluoro-phospho-compounds is shown in Table 2. It is noteworthy that for all three nucleotide analogues the direct phosphorus-fluorine coupling constant is identical, and also that all three show similar upfield shifts due to the substitution. The behaviour of the monofluorophosphate is

Table 3.2

Coupling constants and change in chemical shift upon fluorine substitution

Compound	pH	JP-F (Hz)	Change in chemical shift (ppm)
ATPγF	9.0	936	11.5
ADPβF	9.0	935	12.0
AMPF	9.0	933	10.0
Monofluorophosphate	1.5	910	4.5
	8.0	865	1.6

different, in which the substitution leads to a smaller upfield shift. This is parallelled by thiophosphate, where a smaller effect was observed than for the thio-adenine nucleotide analogues (Jaffe and Cohn, 1978). From the pH-dependency of the signal it appears that protonation causes it to shift upfield by about 5.5 ppm, while orthophosphate shifts the same direction but just over 3 ppm, and thiophosphate shifts downfield over 9.5 ppm due to protonation. Since the overall charge has no effect on the chemical shifts (Van Wazer and Letscher, 1967) the reasons for these differences in behaviour are not clear.

C. pH studies of the methylene analogues. The pKa for the terminal phosphate of ATP is approximately 7.0 and the presence of Mg^{2+} ions lowers this by about 1.5 units, indicating that under physiological conditions the nucleotide is fully deprotonated. Since Yount (1975) has shown that AMPPCP(β,γ) has a pKa >8.0, it was suggested that it is not a good analogue of ATP for *in vitro* tests. In this section we describe a systematic study of the three available methylene-analogues of adenine nucleotide, in the presence and absence of Mg^{2+} ions.

The results for AMPPCP(β,γ) AMPCPP(α,β) and AMPCP(α,β) are shown in Figs. 2, 3 and 4 and in Appendix 1, respectively. When performing these titrations we noted that the protonated and deprotonated forms were in fast exchange since no broadening of resonances was observed. The

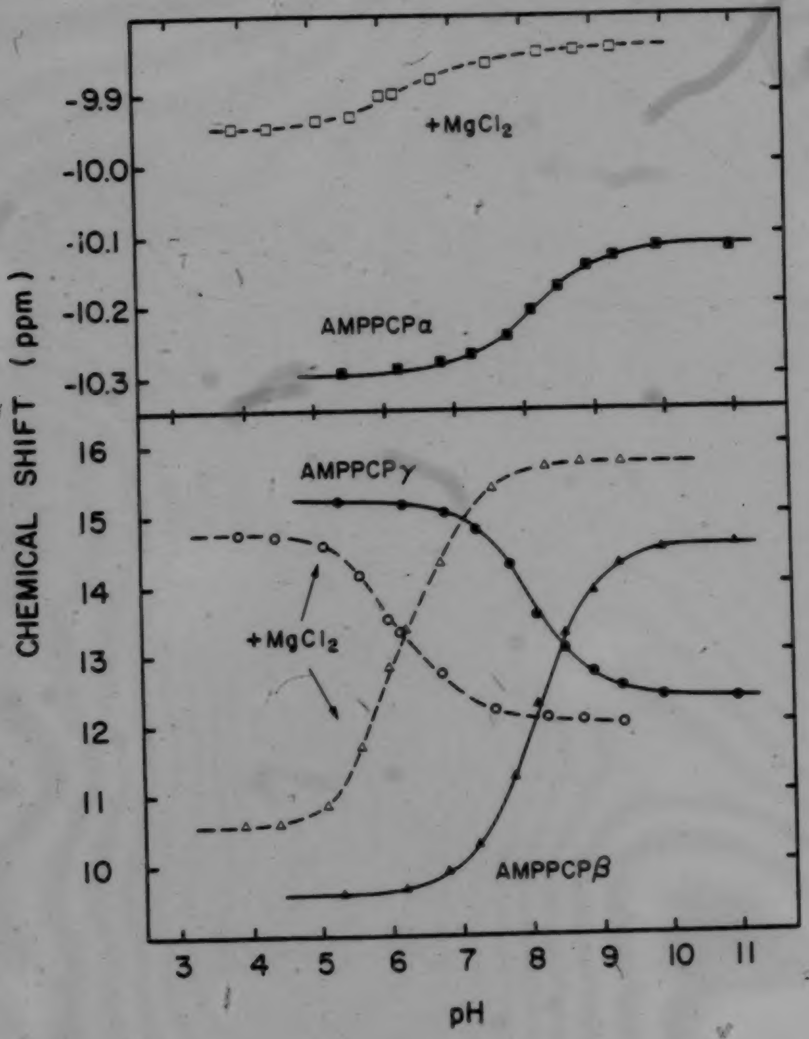

Fig. 3.2 Effect of pH on the chemical shifts as measured in the ^{31}P-NMR spectrum of AMPPCP(β,γ). Solid lines and closed symbols are used for results in the absence of Mg^{2+}, dotted lines and open symbols are used in the presence of Mg^{2+} (5:1) ratio). α-phosphates (■, □), β-phosphates (▲, △), and γ-phosphates (●, ○). Note the different y-axes.

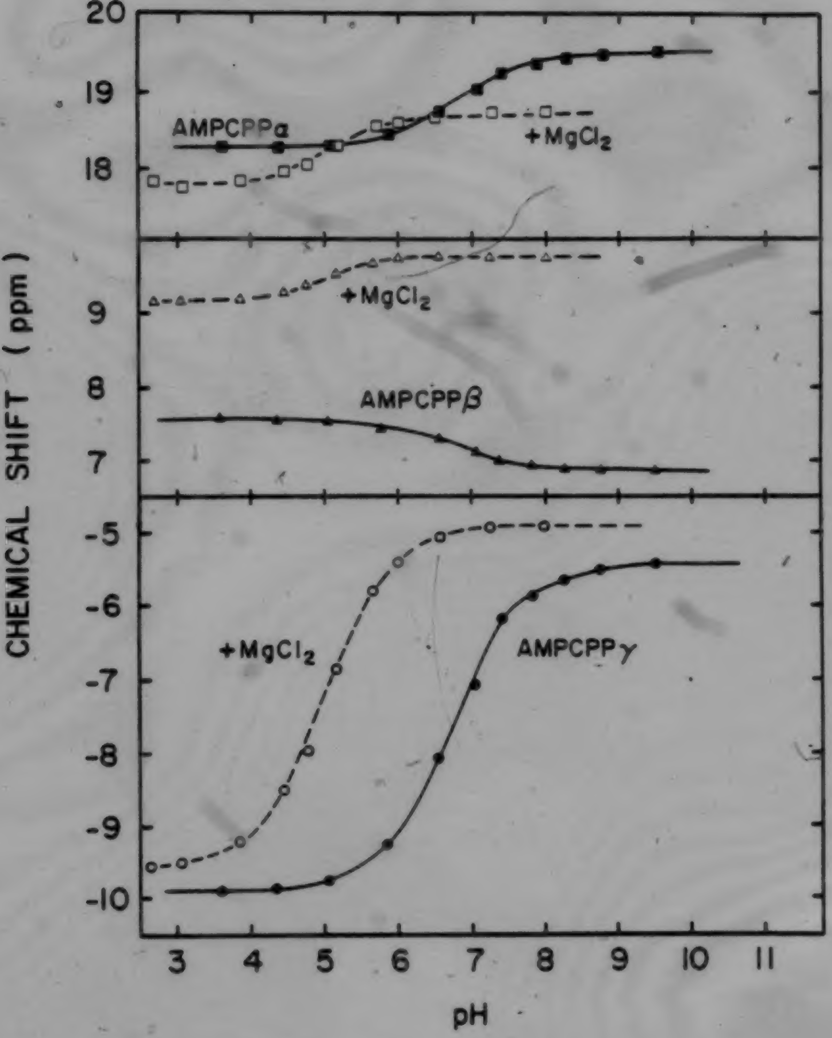

Fig. 3.3 Effect of pH on the chemical shifts as measured in the ^{31}P-NMR spectrum of AMPCPP(α,β). Symbols as in figure 2.

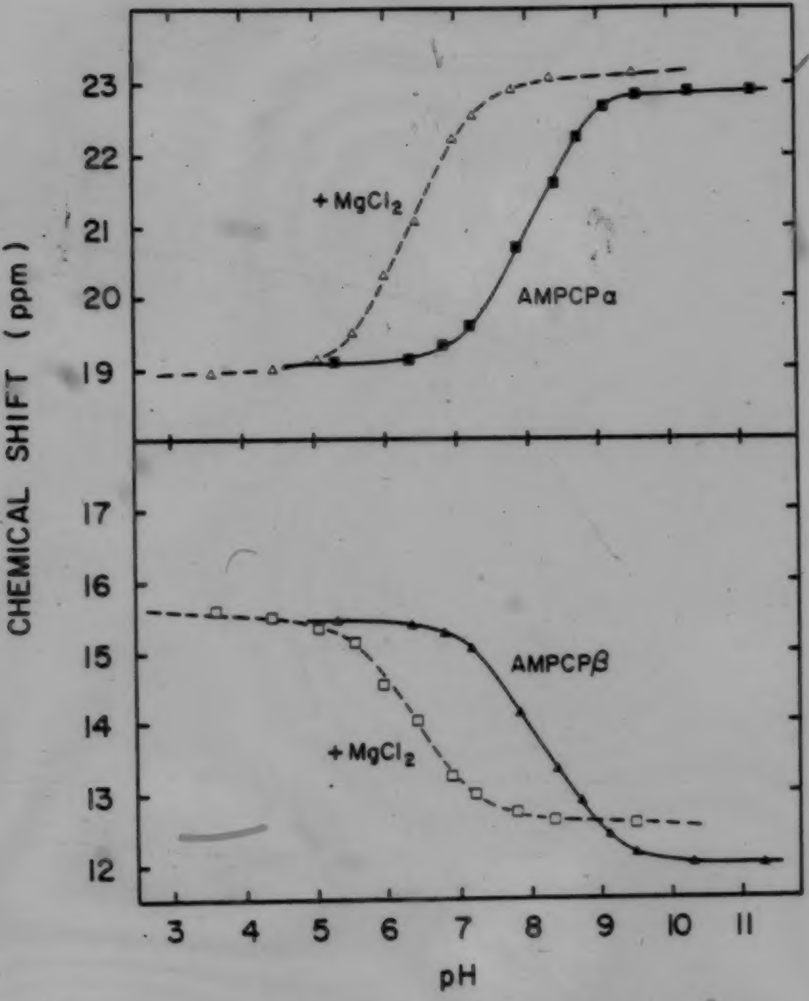

Fig. 3.4 Effect of pH on the chemical shifts as measured in the ^{31}P-NMR spectrum of AMPCP(α,β). Symbols as in figure 2.

Table 3.3

Effect of pH titration and Mg $^{+}$ on NMR parameters and pK_a values of ATP and ADP analogues

Compound	Ref.[a]	ppm (-H^+) - ppm (+H^+)			coupling constants (Hz)		pK_a	ΔpK_a
		α	β	γ	αβ	βγ		
ATP	1,2	0.28	1.39	5.05	19.2	19.2	6.7	1.4
ATP + $MgCl_2$		0.59	2.89	5.04	16.2	15.6	5.3	
AMPPNP (β,γ)	2	0.53	4.01	1.43	20.2	5.5	7.7	1.5
AMPPNP + $MgCl_2$		0.36	3.91	1.34	17.3	9.4	6.2	
AMPPCP (β,γ)	4	0.17	5.0	-2.80	26.5	8.0	8.0	1.8
AMPPCP + $MgCl_2$		0.13	5.15	-2.75	23.0	6.0	6.2	
AMPCPP (α,β)	4	1.3	-0.70	4.5	8.5	25.0	6.8	1.7
AMPCPP + $MgCl_2$		0.95	0.60	4.45	5.5	21.0	5.1	
ATPγS	1	0.15	1.30	-4.5	19.6	29.0	5.3	>1.1
ATPγS + $MgCl_2$		N.D.[b]			15.5	25.4	<4.2	
ATPαS	1	-1.0	1.40	5.0	27.5	20.5	N.D.	
ATPγF	4	N.A.[c]			19.5	17.8	N.A.	
ADP	3	0.50	4.60		21.7		6.8	1.5
ADP + $MgCl_2$		1.20	4.60		17.9		5.3	
AMPCP (α,β)	4	3.8	-3.5		9.0		8.1	1.7
AMPCP + $MgCl_2$		4.15	-3.0		3.5		6.4	
ADPβS	1,4	0.30	-4.5		31.2		5.2	>1.0
ADPβS + $MgCl_2$		N.D.			28.1		<4.2	
ADPβF	4	N.A.			19.8		N.A.	

[a] References listed after Table 4.

[b] N.D. = not determined.

[c] N.A. = not applicable.

titration data clearly lead to curves with one inflection and are not as complex as the ones recently published for phosphonic acids (Deslauriers *et al*., 1980). From a comparison of the data for the different resonances of one analogue, it is obvious that all three resonances are affected by titration of a group with the same pKa. Calculation of the Hill coefficients for these data according to the formulas outlined by Markley (1975) gave values in the range of 0.8-1.0.

In Table 3 an overview of the results is presented, including a comparison with ATP, ADP and other analogues.

Comparison of pKa's. While it is clear that the pKa for AMPPCP(β,γ) is higher than that of ATP, in the presence of Mg^{2+} at pH >7 the analogue will be almost completely in the deprotonated form. Results with AMPPNP were very similar (Tran Dinh and Roux, 1977). The situation with the ADP analogue AMPCP(α,β) is similar. It is interesting that the (α,β) methylene analogue of ATP has a titration behaviour almost identical to ATP, consistent with the idea that the pKa is changed only by substitutions on the terminal phosphate. Data obtained with the thiophosphates suggest that this applies equally to nonbridging substitutions with sulfur, since ATPβS has a pKa virtually identical to that of ATP (Jaffe and Cohn, 1978). Although the pKa's for all of the components cover a range, all are changed by ± 1.7 pH units due to Mg^{2+} complexation.[1] This suggests that each of these compounds must complex Mg^{2+} via the terminal phosphate since only competition between Mg^{2+} and H^+ could give rise to this change in pKa.

Comparison of coupling constants. A comparison of all the $J\alpha\beta$ and $J\beta\gamma$ coupling constants (Table 3) shows that for unsubstituted residues the change in coupling constants are normally minimal. Substitution leads to changes (e.g., the P-C-P linkage gives a coupling constant of about 8.5 Hz) and this figure does not appear to depend on the position of the substitution. This value is comparable with that observed for the P-N-P linkage. (5.5 Hz), both are much smaller than the P-O-P linkage (19.5 Hz) coupling constant. Nonbridge fluorosubstitution leads to a small decrease of almost 2 Hz in the P-O-P coupling, whereas nonbridge sulfur leads to an almost 10 Hz increase in the coupling constant.

Most noteworthy is the fact that in every case except AMPPNP(β,γ) the coupling constants for all $J\alpha\beta$ and $J\beta\gamma$ measured decrease by appromixately 3 ppm upon complexation of Mg^{2+}. This is also consistent with a similar mode of Mg^{2+} complex formation with all compounds, and it suggests that all three phosphate residues (or all two with ADP) are involved in the binding.

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[1] Since Mg^{2+} competes with a proton, the magnitude of the pKa may be further affected at higher Mg^{2+} concentrations. The results reported here were obtained with a Mg^{2+}:nucleotide ratio of 5, and like the other data summarized in Table 3, presumably represent the 1:1 complex.

pH titrations: Direction of chemical shifts. The data of Table 3 show that in most cases protonation of the terminal phosphate leads to an upfield shift for all resonances. All thiophosphates, however, show a downfield shift upon protonation, and some (but certainly not all) phosphates linked to a methylene group show similar behaviour. For example, Fig. 2 shows that the β and γ peak of AMPPCP(β,γ) shift in different directions and actually cross each other. Similarly the α-P and β-P of the ADP analogue AMPCP(α,β) shift different directions due to protonation (Fig. 4). Moreover, the β-P of AMPCPP(α,β) normally shows a downfield shift upon protonation, but in the presence of Mg^{2+} an upfield shift is measured (Fig. 3). Such behaviour cannot be explained by effects on electron-distribution only, since methylene substitution would then presumably result in a similar shift direction for all methylene substituted phosphates. Therefore we conclude that the changes observed in the chemical shifts are primarily due to changes in conformation on the polyphosphate chain.

pH titration: Magnitude of change in chemical shifts. On first sight it would seem likely that protonation at the terminal phosphate would produce largest changes in the chemical shift of that resonance. In fact the titration behaviour of ATP and ADP are consistent with this. However, the data for AMPPCP, AMPPNP and AMPCP clearly indicate that it is not the terminal but its neighbouring phosphate that shows the largest change in chemical shift (Table 3, Fig. 2, 3, 4). This further substantiates our earlier conclusion that the changes in chemical shift observed by changes in pH are mainly due to changes in structure. Another effect is noteworthy. Methylene substitution leads in certain cases to a larger magnitude of the change observed for certain residues [e.g. for the α-P of AMPCPP(α,β) and AMPCP(α,β) the change is roughly four times as large as for the corresponding residues of ATP and ADP (see Table 3)]. Similar effects are found for sulfur substitution: compare ATPαS and ATP, for example. Thus, in certain ^{31}P-NMR studies the higher sensitivity of the methylene analogues can be used to advantage. A comparison of the effects of Mg^{2+} on the magnitude of the shift difference due to protonation shows that in some cases Mg^{2+} binding has resulted in large changes. This is clearly evident for the α-P of ADP, ATP and AMPCPP(α,β) and for the β-P of these last two compounds. These effects could reflect direct coordination, but also suggest that Mg^{2+} binding, like protonation, can produce structural rearrangements in the polyphosphate chain.

D. Mg^{2+} titration of fluoro-analogues. Since the pH and the ionic strength were kept constant in these experiments, observed changes in the chemical shifts are solely attributable to complexation by Mg^{2+} ions. Plots of chemical shift versus Mg^{2+} showed a normal saturation curve indicative of 1:1 binding to ADPβF. Reciprocal plots of Mg^{2+}

concentration versus change in chemical shift suggested a Kd of approximately 40 mM.

The titration curves for ATPγF were more complex and did not allow unequivocal distinction between one or two binding sites per nucleotide. The data indicate a single Kd of 8 mM, so if two binding sites exist they must have identical Kd's. This value compares well with the value of 5 mM reported by Yount (1975). The low binding constants observed for these two analogues as compared to those reported for ATP and ADP point to the importance of the terminal phosphate in metal binding.

E. Mg^{2+} titration of methylene analogues. The Mg^{2+} titrations of the ATP analogues AMPPCP(β,γ) and AMPCPP(α,β) and the ADP-analogue AMPCP(α,β) are shown in figures 5-7 and Appendix 1, respectively. All three titrations were performed at pH 7.2, which presents a problem for direct comparison since the three pKa's are different, namely 8.0, 6.8 and 8.1 respectively. However, our objective was to shed light on the mode of Mg^{2+} binding at physiological pH.

When performing these titrations it was apparent that the extreme fast-exchange situation did not prevail. Resonances did not only shift but broadened drastically, but in none of the cases did we observe a split in the peak indicative of slow exchange conditions. We also noted that the largest linewidth usually occurred at less than half

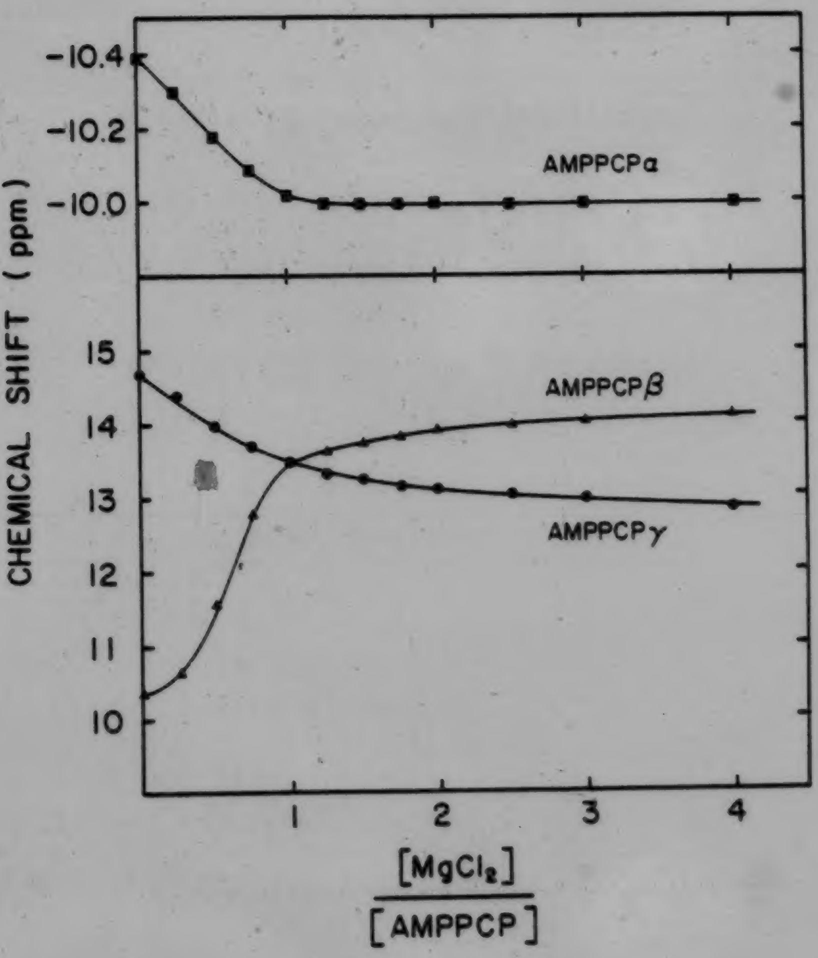

Fig. 3.5 Effect of Mg^{2+} concentration on the chemical shifts as measured in the ^{31}P-NMR spectrum of AMPPCP(β,γ). Conditions of experiment see Materials and Methods. Symbols as in figure 2. Note the different y-axes.

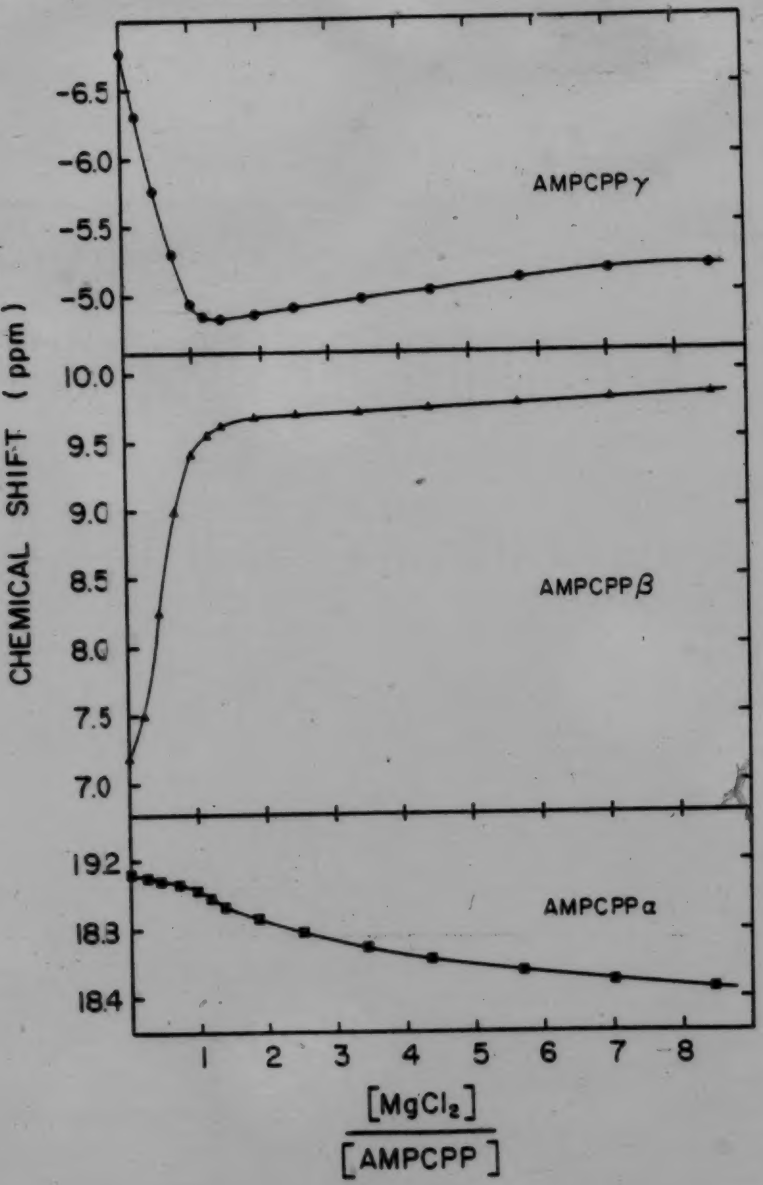

Fig. 3.6 Effect of Mg^{2+} concentration on the chemical shifts as measured in the ^{31}P-NMR spectrum of AMPCPP(α,β). Note the different scale on the x-axis as compared to Fig. 5 and Fig. 7. Symbols as in figure 2.

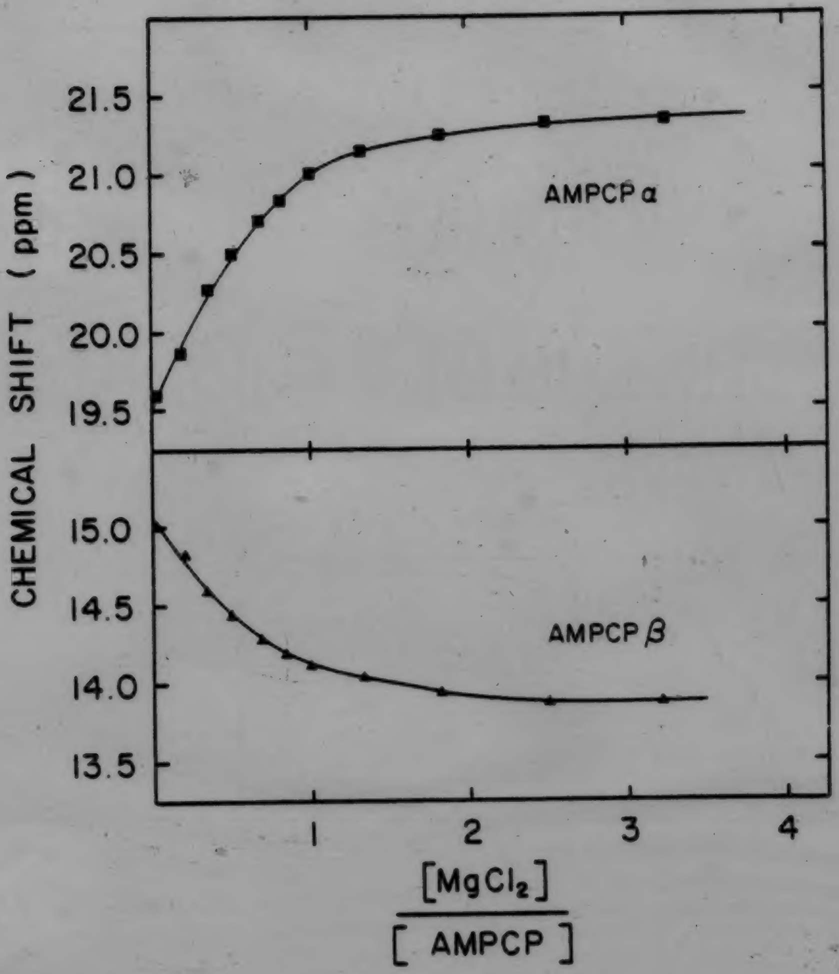

Fig. 3.7 Effect of Mg^{2+} concentration on the chemical shifts as measured in the ^{31}P-NMR spectrum of AMPCP(α,β). Symbols as in figure 2.

saturation. When only one Mg^{2+}-bound and a Mg^{2+}-free form are in exchange, the broadest line would be expected at exactly half saturation. Bishop *et al.* (1981) noted a similar behaviour for ATP, and attributed this to the existence of several coordination structures in solution. This asymmetry in the titration can also be seen especially in the onset of the titration curves, appearing most clearly in the β-P of both ATP methylene analogues but present in the other resonances as well (see Figs. 5,6,7).

The shapes of the titration curves show that all three analogues have one tight binding site. This kind of curve does not allow for calculation of Kd's. (From the shape of the curves it appears that the ADP analogue does not bind Mg^{2+} as tightly as the ATP analogue.) Both ATP analogues, but not the ADP analogue, seem to bind a second Mg^{2+} ion at higher Mg^{2+} concentrations. This is most clearly seen in the curves shown for the γ- and α-P's of AMPCPP(α,β), but is also evident in the curves for the AMPPCP(β,γ) analogue. The binding of the second Mg^{2+} is certainly not as tight as the first with saturation occurring at a ratio of $MgCl_2$/AMPCPP(α,β) of 8. A similar saturation of the second Mg^{2+} binding site was recently observed for ATP (Bishop *et al.*, 1981) with a Kd of 28 mM. Furthermore, Granot *et al.* (1979) have shown that Mn^{2+} binds at a second site to the stable β,γ-bidentate $Co^{3+}(NH_3)_4$ATP complex with a Kd of 15 mM, thus providing direct evidence for binding of two metal ions per nucleotide molecule.

Table 4 summarizes the Mg^{2+} complexation data at pH's where only species with the deprotonated terminal phosphate are present. It can be seen that complexation usually leads to a downfield shift, but upfield shifts are also noted, in keeping with our earlier conclusion that the chemical shift is primarily sensitive to conformational changes in the polyphosphate chain rather than to charge (Ramirez and Mariceck, 1980). Clearly the effects on the β-P of ATP and its analogues are usually largest, which has been interpreted as binding of Mg^{2+} solely to that phosphate. (Tran Dinh *et al.*, 1975). However, this is probably more indicative of the role of pivot that the β-P plays in conformational rearrangements of the poly-phosphate chain. Again, we notice the larger sensitivity of certain thio and methylene compounds to Mg^{2+} complexation.

Discussion

Use of analogues. Methylene compounds can be readily identified by their characteristic chemical shifts in a ^{31}P-NMR spectrum. This has led to their recent *in vivo* discovery in *Acanthamoeba castellanii* (Deslauriers *et al.*, 1980). This same property can be exploited in studies of enzyme mechanisms. For example Hoerl *et al.* (1979) used a methylene-analogue of pyridoxal phosphate in glycogen phosphorylase in order to observe it without interference from

Table 3.4

Change in chemical shift resulting from Mg^{2+}-complexation

Compound	Ref.	pH	ppm ($+Mg^{2+}$) - ppm ($-Mg^{2+}$)		
			α	β	γ
ATP	1	8.0	0.20	2.20	0.50
AMPPNP (β,γ)	2	10.0	0.15	1.10	-0.80
AMPPCP (β,γ)	4	10.0	0.25	1.20	-0.30
AMPCPP (α,β)	4	9.0	-0.85	2.90	0.45
ATPγF	4	8.0	0.20	+0.20	-0.50
ATPγS	1	8.4	0.00	1.40	1.80
ATPβS	1	8.0	0.20	6.10	0.20
ATPαS	1	8.0	2.00	1.60	0.30
ADP	3	10.0	0.70	0.10	
AMPCP (α,β)	4	10.0	0.20	0.60	
ADPβF	4	8.0	-0.80	-0.30	
ADPβS	1,4	8.0	0.10	1.30	

References: (1) Jaffe and Cohn (1978); (2) Tran Dinh and Roux (1977); (3) Tran Dinh and Neumann (1977); (4) This report.

other phosphomonoesters. Schnackerz and Feldman (1980) used the same analogue, since its relatively high pKa allowed pH titrations without affecting the stability of serine-dehydratase. The majority of ^{31}P-NMR studies on nucleotide binding to enzymes and proteins have been done using ATP and ADP, without use of these analogues (for review see Cohn and Rao, 1980). AMPPNP(β,γ) was recently used in a study with myosin (Shriver and Sykes, 1981). In a ^{31}P-NMR study of succinyl-CoA synthetase, (Vogel and Bridger, 1982), where we wished to study ATP binding and the covalent phosphohistidine residue simultaneously we used AMPPCP(β,γ) since ATP and other analogues had resonances overlapping with the phosphohistidine. In this study we observed a large chemical shift difference between free and bound AMPPCP, much larger than the ones reported for ATP and ADP (Cohn and Rao, 1980). That result is in agreement with our observations here, since we have shown here that the chemical shifts of methylene analogs are generally more sensitive towards changes in protonation (Fig. 2, 3, 4; Table 3) and Mg^{2+} binding (Fig. 5, 6, 7; Table 4). Similarly, the thio-nucleotides show a greater sensitivity (Jaffe and Cohn, 1978).

The pH-titration studies might suggest that both AMPPCP(β,γ) and AMPCP(α,β) have an unsatisfactorily high pKa for the terminal phosphate to be good analogues, but in the presence of saturating amounts of Mg^{2+} ions the pKa's are sufficiently low that these analogues will mimmic ATP and

ADP at physiological pH. The other analogue AMPCPP(α,β) is virtually identical to ATP in terms of titration behaviour (see Table 3). In terms of Mg^{2+} binding both ATP analogues can bind two Mg^{2+} (one tight, one loose) in similar fashion to ATP (Bishop *et al.*, 1981). The ADP analogue AMPCP(α,β) like ADP, binds only one Mg^{2+}. These conclusions do not support the suggestion (Yang *et al.*, 1981) that different modes of metal binding could account for different activation properties of phosphatases by ATP and its analogues.

The fluorine-analogues may have a more limited use because of their poor complexation of Mg^{2+}. As pointed out earlier, these analogues can be advantageously used to probe the specificity binding sites for either mono- or dianions (Yount, 1975; Withers and Madsen, 1980), but in the absence of Mg^{2+} also AMPPCP(β,γ) and AMPCP(α,β) could be used. Perhaps the large *JPF* (935 Hz) can become a convenient parameter to monitor changes in environment. The higher sensitivity and shorter T_1's of ^{19}F NMR could facilitate the use of these analogues.

Since the chemical shifts of these fluoro analogues are clearly pH-independent, this confirms that the adenine ring titratable groups do not influence the structure of the polyphosphate chain.

Chemical Shifts. Early interpretations of the ^{31}P-NMR spectrum of ATP accounted for the most upfield position of the β-P of ATP by the higher shielding by electrons for this nucleus than for the other two. The downfield shift observed upon addition of Mg^{2+} then would signal a deshielding (Mildvan, 1970). However, the results of Table 3 show that Mg^{2+} complexation can lead to both downfield and upfield shifts, indicating that the situation is more complex than that electronegative substituents "withdraw electrons" and therefore deshield the P-nucleus to result in a downfield shift. In our studies of analogues we noted that for nonbridging substitutions the value of chemical shift increased going from S > O > F; likewise for the bridging substitutions the order found was C > N > O (Table 1). This result is exactly opposite to that predicted on the basis of the deshielding rationale. Van Wazer and Letscher (1967) have noted this anomaly earlier. From quantum mechanical considerations they concluded that the observed deshielding is a function of three interdependent parameters - electronegativity, bond angle and amount of bonding. More recently Gorenstein *et al.* (1976) showed an empirical corelation between bond angle and chemical shift, perhaps resulting from an effect of geometry on σ and π electron density on the phosphorus nucleus. This poses the experimentalist with the problem of one measurable parameter (chemical shift) and three unknowns. This dilemma could only be solved if other parameters (e.g. coupling constants, T_1, T_2) could be measured and their relationships with the three unknowns were delineated. Unfortunately, this is not the case, and interpretations of ^{31}P-NMR spectra remain largely empirical. Furthermore, since the interplay of contributing factors is still not understood, failure to observe a change in chemical shift is not of itself evidence against chemical or structural modification of a given nucleus.

Table 1 shows that the chemical shifts change only for nuclei that are directly substituted, consistent with the idea that substitution does not produce gross differences in the structure of these compounds.

Metal binding. The similarity in the decrease in coupling constant upon Mg^{2+} binding to all analogues is in keeping with a similar mode of metal complexation to all of them, as is the similarity in the decrease in pKa for all analogues upon Mg^{2+} complexation. These considerations, together with our observations that the fluoro-analogues bind Mg^{2+} poorly, support the idea that the terminal phosphate must be important in metal binding. Since the $J\alpha\beta$ as well as the $J\beta\gamma$ on the ATP analogue change similarly, and since we have observed asymmetry in the Mg^{2+} titration curves, we favour a model in which all possible mono-, bi- and tridentate coordination structures exist in solution in fast equilibrium, with the interconversion between the different coordination structures faster than the Mg^{2+} on and off rates. Thus, the ^{31}P-NMR spectrum would be the fast exchange average of these structures. Similar suggestions were recently made for the structures of ATP and ADP

(Cleland and Mildvan, 1979; Ramirez and Mariceck, 1980). Since the use of thiophosphate- and exchange-inert analogues has indicated that most enzymes show strong preferences for specific coordination structures, one can visualize a situation in which the different coordination structures are all in fast equilibrium in solution, and an enzyme can select the one it specifically recognizes (Cleland and Mildvan, 1979; Mildvan, 1979; Eckstein, 1980). Unfortunately, however, this also means that the ^{31}P-NMR spectra of solution and enzyme-bound Mg-ATP and Mg-ADP cannot be directly compared, since this would require knowledge of the chemical shifts for all different coordination structures in solution, in order to resolve contributions to the chemical shift caused by binding to the enzyme. Different chemical shifts for the various coordination structures have been reported only for the Cobalt exchange-inert complexes of ATP (Cornelius *et al.*, 1977). We conclude that it is valid to use Mg^{2+} induced shifts in ATP and ADP for measuring the degree of complexation *in vitro* and *in vivo*, assuming that the on and off rates and interconversion rates will be similar in both cases.

Another approach to determine the structure of the adenine-nucleotide polyphosphate ATP-Mg complex in solution has been the study of the changes in relaxation properties due to titration with paramagnetic metal ions (Cohn and Hughes, 1962; Brown *et al.*, 1973; Tanswell *et al.*, 1975).

The results of these studies placed the metal ion closer to the β- and γ- than the α-P. Although this could be easily accommodated in the interpretations presented here by assuming that the equilibrium of all coordination structures is not random, it must be borne in mind that quite different binding constants have been observed for different metal ions (Yount, 1975). It may thus be dangerous to extrapolate the results with paramagnetic metals to the Mg-ATP complexes.

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CHAPTER 4

FREQUENCY DEPENDENT ^{31}P-NMR STUDIES OF THE PHOSPHOHISTIDINE RESIDUE OF SUCCINYL-COENZYME A SYNTHETASE AND THE PHOSPHOSERINE RESIDUE OF GLYCOGEN PHOSPHORYLASE-*a*

Summary

^{31}P-NMR spectra of the active site N-3 phosphohistidine residue of *Escherichia coli* succinyl-CoA synthetase and the phosphoserine residue on the regulatory site of rabbit muscle glycogen phosphorylase *a* were obtained at four different magnetic field strengths. Plots of observed linewidth versus the square of the resonance frequency allowed us to differentiate between relaxation caused by field-independent dipolar terms and field-dependent chemical shift anisotropy terms. From this last contribution we calculated for *E. coli* succinyl-CoA synthetase and for the N-1 phosphohistidine residue of *E. coli* HPr that the phosphorus atom is rigidly held and has no residual mobility on the protein. For the phosphoserine residue of glycogen phosphorylase, we deduced that exchange takes place between two conformations. In general it seems that the phosphorylated residues that are catalytic intermediates in phosphoryl transfer reactions are immobilized, whereas the phosphoserine residues at regulatory sites have some mobility. Moreover, from the difference in the dipolar terms observed for succinyl-CoA synthetase (11 Hz) and glycogen phosphorylase (6 Hz) we

deduced that the phosphoryl group of the N-3 phosphohistidine residue is in the monoanionic form and that the phosphoserine residue is in the dianionic form at pH 7.25. This observation is consistent with the pKa's that have been reported for phosphoserine and for phosphohistidine.

For both the N-3 and N-1 phosphohistidine residues of succinyl-CoA synthetase and HPr, we have calculated an anisotropy term $\Delta\sigma(1 + \eta^2/3)^{1/2} = 230$ ppm. This value is higher than those that we determined from powder spectra of comparable model compounds. Explanations for this discrepancy are considered, including the intriguing possibility that the geometry of phosphorylated intermediates of phosphotransferases is changed to facilitate in-line nucleophilic attack of the phosphoryl group.

Introduction

^{31}P-NMR has recently become a popular tool for studying the binding of phosphorus-containing ligands to enzymes and other proteins (Cohn and Rao, 1979). In addition this technique allows for non-invasive study of covalently bound coenzymes and phosphoamino acids on proteins. Typical examples of coenzymes that have been studied in this way are FAD on glucose oxidase (James *et al.*, 1980) and some pyridoxal-phosphate containing enzymes, most notably glycogen phosphorylase (Feldmann and Hull, 1977; Withers *et al.*, 1981). These studies have provided insight about the environment of the P-atoms of these coenzymes. Unfortunately, however, no theory has yet been formulated to translate the observed chemical shifts into molecular details.

Enzymes and other proteins carrying phosphoamino acids can be subdivided into two large groups. The first one is that of the phosphoryl-transferring enzymes, the majority of which catalyze a direct transfer of a phosphoryl group from a phosphate donor (e.g. ATP) onto another substrate. Some phosphotransferases, however, become transiently phosphorylated on a specific amino acid residue; this phospho-enzyme form can thus serve as an intermediate between phosphoryl donating and accepting substrates (Knowles, 1980). Amino acid side-chains that have been reported to be phosphorylated in this way include those of serine and histidine residues (Bridger, 1979). Examples of enzymes with phosphoserine intermediates that have been studied by ^{31}P-NMR are *E. coli* alkaline phosphatase (Chlebowski *et al.*, 1976; Coleman and Chlebowski, 1979; Hull *et al.*, 1977) and phosphoglucomutase (Ray *et al.*, 1977). The only phosphohistidine-containing protein studied until now is the HPr phosphoryl carrier protein of the bacterial glucose transport system (Gassner *et al.*, 1977, Dooijewaard *et al.*, 1979). This protein is atypical in that it carries the phosphoryl group on the N-1 of the histidine instead of on the N-3 where it has been most frequently found (Bridger, 1973). A good representative of this class is *E. coli* succinyl CoA synthetase (Bridger, 1974). second group of phosphoproteins comprises those whose biological activity is regulated by phosphorylation. Unique serine residues usually remote from the active site are phosphorylated and dephosphorylated by specific protein kinases or phosphatases whose activity is under hormonal control (Krebs and Beavo, 1979). Glycogen phosphorylase, for example, becomes markedly activated upon phosphorylation of its serine-14 residue (Fletterick and Madsen, 1980).

It has been demonstrated for a variety of large macromolecular structures that the relaxation of ^{31}P nuclei is dominated by chemical shift anisotropy (CSA), leading to large increases in the observed linewidth especially at high field strengths. This effect has been observed for phospholipid vesicles (Berden *et al.*, 1974), bacteriophages (Opella *et al.*, 1981) and in DNA (Shindo, 1980). Since this mechanism also prevails for relatively small macromolecules such as transfer-RNA (Guéron and Shulman, 1975) and actin (Brauer and Sykes, 1981), we were prompted to look for this same phenomenon in phosphoproteins. In this communication we report frequency-dependent ^{31}P-NMR studies of the N-3 phosphohistidine residue of *E. coli* succinyl-CoA synthetase and the phosphoserine residue of glycogen phosphorylase *a*, leading to the clear demonstration that CSA is the dominant relaxation mechanism at high field strengths. A similar

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conclusion is reached for the N-1 phosphohistidine residue of HPr. The linewidths are further analyzed in terms of the mobility of the protein-bound phosphoryl groups.

Materials and Methods

Succinyl-CoA synthetase was purified from Crooke's strain of *E. coli* grown on succinate-based medium according to methods previously described (Wolodko *et al.*, 1980). Activities were measured and NMR samples were prepared as previously described (Buttlaire *et al.*, 1977). The specific activity was checked before and after NMR spectra were taken and was never below 35 units per mg of protein. NMR samples contained 25 mg/ml enzyme in 40 mM Tris (ultrapure), 80 mM KCl, 25% D_2O (Biorad), pH 7.2 (meter reading). Phosphorylase was purified from rabbit skeletal muscle and was a gift from Dr. N.B. Madsen and Mrs. S. Shechosky. Samples were prepared as described by Withers *et al.* (1981); typically a sample of 35 mg/ml of phosphorylase *a* was used in a buffer of 100 mM Tris, 2.5 mM DTT, 1 mM EDTA, 50% D_2O, pH 7.3. All buffers were passed through a column of Chelex 100 (Bio-Rad) before use.

^{31}P-NMR spectra were obtained with four different spectrometers, Bruker WH-400, HXS-270, WH-200 and HFX-90 at the Departments of Chemistry and Biochemistry at the University of Alberta. All operated in the Fourier transform mode. Normally a 60° pulse angle was applied and the recycle time for the experiment was 2 sec. All spectra of phosphorylase *a* were proton decoupled. The reported linewidths are corrected for the line broadening caused by computer digital filtering. Samples of 2 ml were in 10 mm Wilmad precision tubes, equipped with Teflon vortex plugs. All experiments were performed at 27°C. ^{31}P-NMR spectra for the powder patterns were obtained at 109.3 MHz without proton decoupling. A pulse angle of 25° was used and the recycle time was 10 ~~sec.~~ The principal elements of the chemical shift tensor were obtained by fitting the observed spectra with calculated powder spectra that were artificially broadened by multiplication with a gaussian function with full width at half height of 4200 Hz. The anisotropy values calculated in this way appear to be approximately 15% overestimated in comparison with literature values (see Results and Discussion). This may result from the fact that dipolar couplings were not removed by proton decoupling.

Creatine phosphate, acetyl phosphate, phosphoserine and 3',5'-cyclic-AMP were obtained from Sigma. Calcium imidazole-diphosphate was synthesized according to the method of Rosenberg (1964).

Theory

Relaxation Theory. Several possible relaxation mechanisms can contribute to the nuclear spin relaxation rates of phosphorus nuclei. Since we are concerned here with phosphates covalently linked to proteins we can assume that chemical exchange does not play a role. Moreover, since spin rotation and scalar relaxation are usually only important for molecules with short correlation times, we will neglect their contributions. Therefore we can confine our calculations here to contributions caused by dipole-dipole interactions between the phosphorus nucleus and neighbouring hydrogen and nitrogen nuclei, and contributions due to chemical shift anisotropy.

1. *Dipole-Dipole Interactions.* The relaxation for a phosphorus atom due to the presence of a hydrogen atom is described (Shindo 1980; Hull and Sykes, 1975) as,

$$\pi.\Delta\nu = 1/T_2 = C[4J(o) + J(\omega_P-\omega_H) + 3J(\omega_P) + 6J(\omega_H) + 6J(\omega_H + \omega_P)] \quad (1)$$

where $C = \frac{1}{30} S(S+1) h^2 \gamma_P^2 \gamma_H^2 r_{PH}^{-6}$ (2)

and the spectral density $J(\omega_i) = \frac{2\tau_c}{1 + (\omega_i \tau_c)^2}$ (3)

and where $\Delta\nu$ = linewidth (Hz), T_2 = spin-spin relaxation time, ω_P and ω_H are respectively the phosphorus and proton frequencies, γi is the gyromagnetic ratio for different nuclei, r_{PH} is the distance between phosphorus and proton nuclei, S is the nuclear spin quantum number, and τ_c is the correlation time. For all proteins and frequencies that we will discuss in this communication, the "nonextreme narrowing limit" condition is fulfilled: $(\omega_P \tau_c)^2$, $(\omega_H \tau_c)^2$,

$[(\omega_P - \omega_H)\tau_c]^2$, $[(\omega_P + \omega_H)\tau_c)^2 > 1$. This reduces equation (1) with substitution of (2) and (3) to

$$\pi.\Delta\nu = \frac{1}{T_2} = \frac{1}{5}\,\frac{\gamma P^2\ \gamma H^2\ h^2}{r^6_{PH}} \cdot \tau_c \qquad (4)$$

Likewise it can be shown that for phosphorus-nitrogen dipole-dipole interaction we find

$$\pi.\Delta\nu = \frac{1}{T_2} = \frac{16}{30}\,\frac{\gamma P^2\ \gamma N^2\ h^2}{r^2_{PN}} \cdot \tau_c \qquad (5)$$

2. *Chemical Shift Anisotropy*. The expression for the chemical shift anisotropy is (Shindo, 1980; Hull and Sykes, 1975).

$$\pi.\Delta\nu = \frac{1}{T_2} = \frac{1}{90}\ \omega_P{}^2(\Delta\sigma)^2(1+\frac{\eta^2}{3})\ [4J(o) + 3J\ (\omega_P)] \qquad (6)$$

For definition of terms see Hull and Sykes (1975). In the nonextreme narrowing limit we find that:

$$\pi.\Delta\nu = \frac{1}{T_2} = \frac{4}{45}\ \omega_P{}^2\ (\Delta\sigma)^2\ (1 + \frac{\eta^2}{3})\ \tau_c \qquad (7)$$

It is clear from equations (4) and (7) that the dipole-dipole contributions are not frequency dependent, whereas the chemical shift anisotropy contribution is a function of the square of the magnetic field. The strategy therefore used in this paper is to plot observed linewidth versus $\omega_P{}^2$;

the y-intercept then gives the linewidth contribution from dipole-dipole mechanisms.

Calculation of overall rotational correlation time for proteins. For globular proteins the radius of spherical molecules can be directly deduced from the molecular weight. $r = (3 M_W \bar{v}/4 \pi N)^{1/3}$ where r is the radius and M_W is the molecular weight, with v being the partial specific volume (0.73 ml/gram) and N is Avogadro's number. This calculation gives a low estimate for the radius since it neglects contributions due to hydration of the molecule. Typical globular proteins are known to have 0.30 - 0.40 grams H_2O bound per gram of anhydrous protein, leading to an increase of about 3.0 Å in the radius of each protein (see Cantor and Schimmel, 1980). For calculations of the overall τ_c we therefore applied this correction. The overall correlation time can be calculated by $\tau_c = (4 \pi \eta r^3)/(3kT)$ where η is the viscosity (0.8513 Cp at 27°C) and T is the temperature (300°K for these experiments). Results for these calculations are shown in Table 1. Note that the calculated τ_c's are probably still low estimates since they are based on the assumption that the proteins are round spheres. For different experimental temperatures different τ_c's have to be calculated, since especially η is strongly temperature dependent.

Table 4.1

Correlation times calculated for several phosphoproteins

Protein	Residue	Molecular weight	Calculated radius (Å) hydrated	Correlation time (nsec)
Phosphorylase *a*				
tetramer	P-ser, PLP	400,000	52	121
dimer	P-ser, PLP	200,000	42	64
Succ-CoA synthetase				
E. coli	P-his (N-3)	140,000	37	44
pig heart	P-his (N-3)	70,000	30	23
HPr, *E. coli*	P-his (N-1)	9,000	17	4.2

Calculations were performed as described in the text, for a temperature of 27°C. The abbreviations are: P-ser, phosphoserine; PLP, pyridoxal phosphate; P-his, phosphohistidine.

Results and Discussion

The N-3 phosphohistidine residue of E. coli succinyl-CoA synthetase. This enzyme carries out the substrate-level phosphorylation in the tricarboxylic acid cycle. Both the prokaryotic and the eukaryotic enzymes are known to possess an N-3 phosphohistidine residue that has been established to be an obligatory catalytic intermediate (Bridger, 1973). Here we report on the *E. coli* enzyme which has an $\alpha_2\beta_2$ structure and a molecular weight of 140,000. The two active sites are each arranged at the interface of an α and β subunit but only one α is phosphorylated at any one time (see Bridger, 1974). In Fig. 1 we show the ^{31}P-NMR spectra obtained at four different frequencies. The chemical shift position determined for the resonance is 4.8 ppm upfield from H_3PO_4. This confirms the phosphorylation position on the histidine, since studies of model compounds have shown that N-3 phosphohistidine gives rise to a resonance at 4.5 ppm, whereas the line for N-1 phosphohistidine is at 5.5 ppm (Gassner *et al.*, 1977). Changes observed in the chemical shift and linewidth of this resonance upon addition of substrates are reported elsewhere (Vogel and Bridger, 1981). The spectra (Fig. 1) show that the linewidth increases markedly with the field strength. This also has a significant influence on the observed signal to noise ratio, which seems optimal at 81.0 MHz. Thus, application of higher frequency instruments for ^{31}P-NMR studies on similar

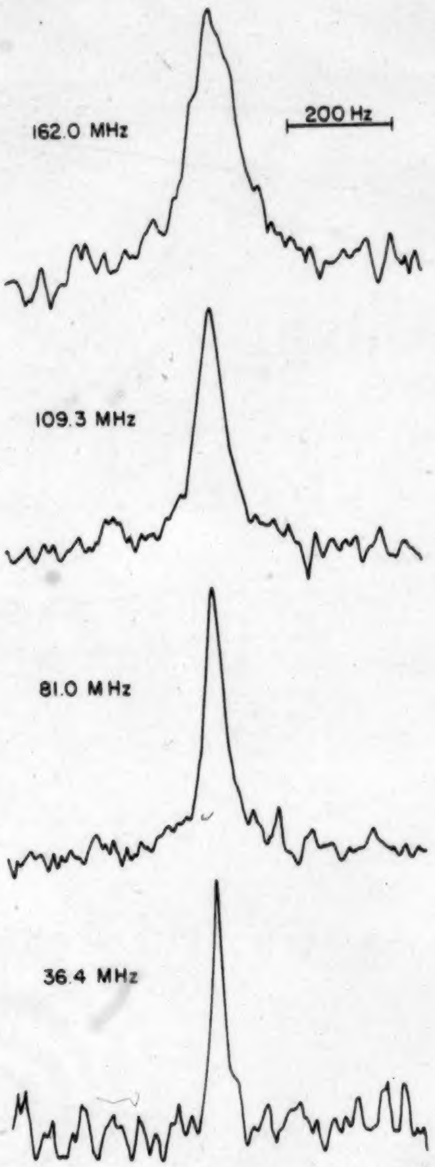

Fig. 4.1 ^{31}P-NMR spectra measured for a sample of *E. coli* succinyl-CoA synthetase (28 mg/ml, pH 7.2). All spectra were obtained as described in Materials and Methods and are plotted with an instrumental line broadening of 10 Hz. Total number of scans collected for each spectrum was: 162 MHz, 27,000 scans; 109.3 MHz, 33,000 scans; 81 MHz, 27,000 scans;, 36.4 MHz, 33,000 scans.

proteins will not necessarily lead to better resolution and signal to noise ratio. This behavior is mainly due to the fact that the chemical shift anisotropy becomes the dominant relaxation mechanism at high frequency.

Fig. 2 clearly shows this dominance of CSA at higher frequency. For example, at 109.3 MHz it contributes 40 Hz to the linewidth which is 75% of the total, at 162 MHz this is increased to 90%. Using equation (7) and substituting the observed linewidth and the correlation time (Table 1) we can calculate the anisotropy term $\Delta\sigma(1 + \eta^2/3)^{1/2}$ = 232 ppm. This value is quite high; values calculated from the elements of the chemical shift tensor for model compounds seldom exceed 170 ppm. This is discussed in more detail in a subsequent section. If we use equation (7) and solve for τ_c, substituting an anisotropy factor of 187 ppm (see Table 2) we obtain a τ_c = 84 nsec. Since this exceeds the overall rotational correlation time theoretically calculated for this enzyme (Table 1), the phosphoryl moiety is clearly tightly bound and has no mobility on the protein surface. Similar calculations show that the dimeric succinyl-CoA synthetase from pig heart would give a resonance with a total linewidth of 30 Hz at 109.3 MHz. field-independent dipole-dipole contribution can be deduced from Fig. 2 to be 11 Hz. In the case of an N-3 phosphohistidine we have to consider several possible contributions to the dipole-dipole interactions (see insert Fig. 2). These are dipolar

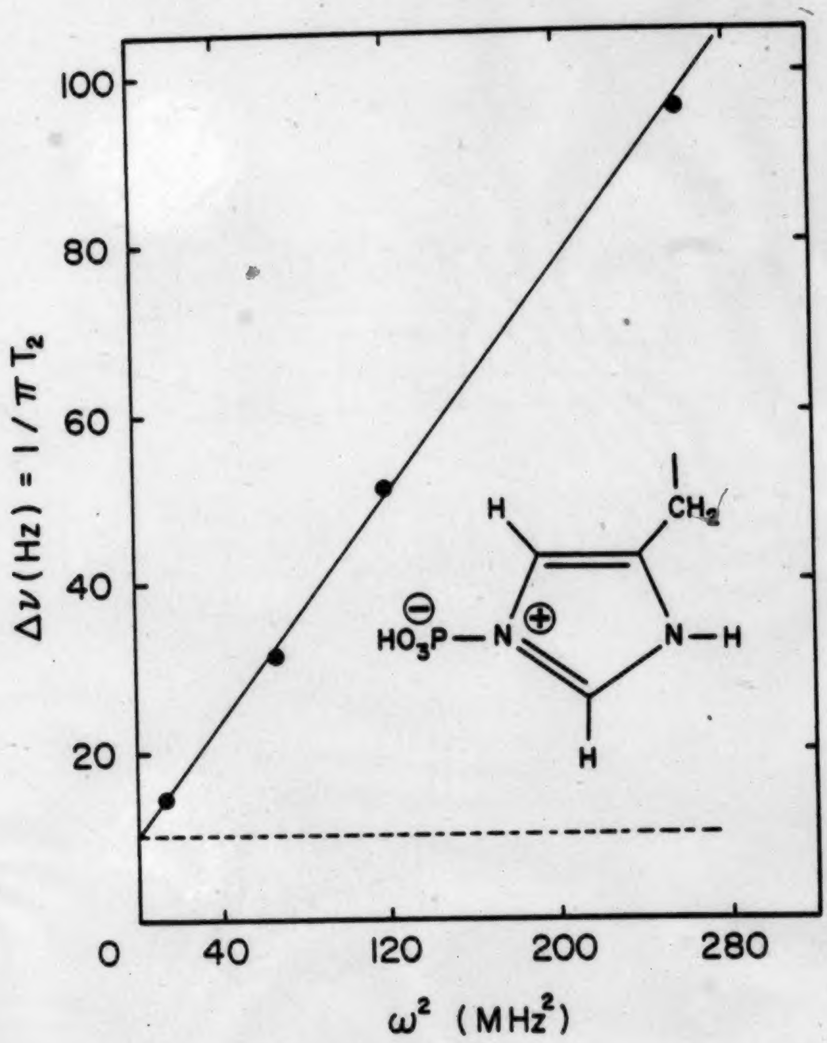

Fig. 4.2 ³¹P-NMR linewidth observed for the N-3 phosphohistidine residue of *E. coli* succinyl-CoA synthetase at four different frequencies. The linewidths were measured at half height and instrumental line broadening was substracted. Maximum estimated error per point was 2.5%.

relaxation due to the two protons on the imidazole ring, protons on the protein that may be hydrogen bonded to the oxygen atoms of the phosphoryl group, a proton covalently attached to one of the oxyanions of the phosphate, or contributions due to the ring nitrogen. These are considered in turn in the following:

1. The two ring protons are both at a distance of 2.9 Å (Beard and Lenhert, 1968). By substitution into equation (4), using the τ_c as indicated in Table 1 one obtains $\Delta\nu$ (ring protons) = 0.90 Hz.

2. The hydrogen-bonded protons are presumably at a distance over 2.9 Å (Sundralingam and Putkey, 1970). Since this experiment was carried out in 25% D_2O this reduces their contributions ($\gamma_D \ll \gamma_H$). We estimate that such protons could maximally contribute 2 Hz to the linewidth.

3. A hydrogen atom covalently linked to one of the oxygen atoms of the phosphate would be at a distance of 1.7 Å (Sundralingam and Putkey, 1970). We calculated (25% D_2O) that this contributes 8.2 Hz to the linewidth.

4. The distance between the phosphorus and nitrogen atoms is 1.8 Å (Beard and Lenhert, 1968). Using equation (5) we find that this contributes $\Delta\nu$ = 0.082 Hz.

Clearly all contributions to the linewidth caused by the dipole-dipole relaxation are small, with the exception of the proton on the phosphoryl group which by itself almost fully accounts for the observed-field independent linewidth. Therefore we propose that the magnitude of the linewidth contribution from dipolar interactions indicates that the N-3 phosphohistidine side-chain is in the monoanion form.

The N-1 Phosphohistidine Residue of E. coli HPr. The bacterial phosphoryl carrier protein HPr possesses one resonance in an ^{31}P-NMR spectrum at 4.1 ppm upfield from 85% H_3PO_4. This position is characteristic for neither N-3 nor N-1 phosphohistidine model compounds. However, denatured HPr shows a resonance at 5.6 ppm characteristic of an N-1 phosphohistidine (Gassner *et al.*, 1977, Dooijewaard *et al.*, 1979). At 145.7 MHz the observed linewidth of native phospho-HPr is 9 ± 1 Hz. These experiments were performed at pH 7.8 and in 100% D_2O (Dooijewaard *et al.*, 1979), so contributions due to exchangeable protons will be negligible. Other dipolar contributions similar to those considered for succinyl-CoA synthetase can also be neglected due to the shorter τ_c for HPr. We also calculated that the new contribution due to the methylene-protons (see insert Fig. 2) for the N-1 phosphohistidine is negligible, so the total linewidth is determined by CSA. We calculated an anisotropy factor of $\Delta\sigma\,(1 + \eta^2/3)^{1/2} = 228$ ppm. Alternatively, the τ_c calculated for the phosphoryl group using an anisotropy factor as determined for model compounds (Table 2) is 11 nsec, longer than that determined for the overall correlation time of the protein. Thus, no residual mobility of the phosphorus moiety on the protein is detected, similar to the result obtained for succinyl-CoA synthetase.

The phosphoserine residue of glycogen phosphorylase. The spectra obtained for this protein showed similar dependence of signal to noise and resolution on field strength as those reported for succinyl-CoA synthetase (data not shown). In the presence of the inhibitor glucose, phosphorylase *a* is in the dimer form and the ^{31}P resonance observed for the phosphoserine residue does not significantly overlap with that of the pyridoxal phosphate (Withers *et al.*, 1981). Fig. 3 clearly shows that in this case the linewidth at high frequency is also mainly determined by field-dependent mechanisms. Recently, Withers *et al.* (1981) showed that the thiophosphoserine residue of phosphorylase *a* has an almost identical linewidth for dimer and tetramer, so it seems that the linewidth of the phosphoserine residue of phosphorylase *a* is more determined by local mobility than by the overall mobility of the protein. From equation (7) we can calculate that the anisotropy term is $\Delta\sigma(1 + \eta^2/3)^{1/2} = 220$ ppm. This clearly is much larger than values found for related model compounds (*ca.* 100 ppm). Since in a mobile case this value would have been lower than those for model compounds, the resonance seen is probably an exchange-broadened average of two different conformations

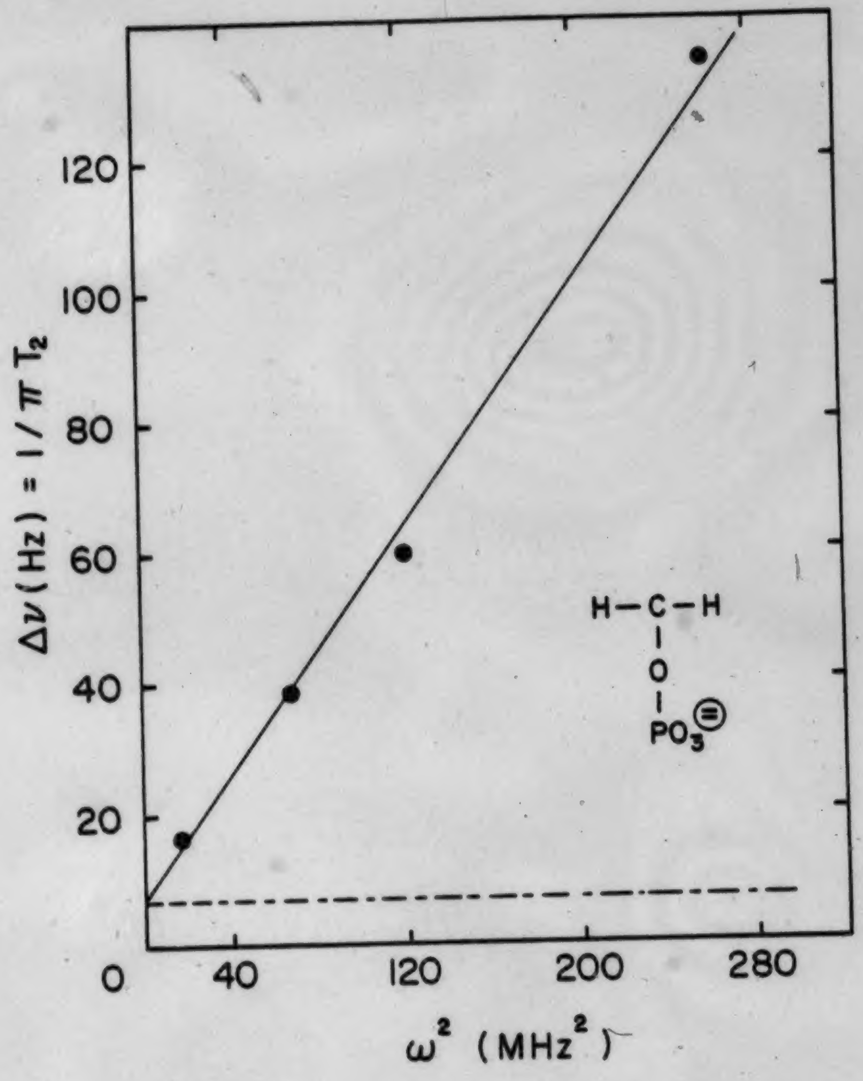

Fig. 4.3 ^{31}P-NMR linewidth observed for the phosphoserine residue of glycogen phosphorylase *a* in the presence of 100 mM glucose. Maximum estimated error per point was 6%.

with different chemical shifts. Since this chemical shift difference itself is a function of ω^2 (Hull and Sykes, 1975), such a process leads to an even larger increase in linewidth at higher fields than can be caused by CSA alone. In pH titration experiments (see Chapter 8), we noted an increase in linewidth with decreasing pH. All these observations support the view that the resulting resonance contains contributions from more than one conformation. Furthermore, Hoerl *et al*. (1979) reported a split resonance for the phosphoserine residue of glycogen phosphorylase *a*.

The field-independent contribution originating from dipole-dipole mechanisms is 6 Hz, less than observed for the N-3 phosphohistidine on succinyl-CoA synthetase. This seems surprising at first, considering the longer τ_c calculated for the phosphorylase dimer (Table 1). For a calculation of the phosphorus-proton dipole-dipole relaxation we have to consider the two nonexchangeable methylene protons that are at a distance of 2.5 Å (see insert Fig. 3) (Sundralingam and Putkey, 1970). Substitution of this distance and a τ_c = 64 nsecs (Table 1) into equation (4) leads to a calculated linewidth $\Delta\nu$ = 3.2 Hz. From X-ray crystallographic data (Fletterick and Madsen, 1980) it is known that the phosphoryl group is surrounded by arginine residues. These contribute hydrogen-bonded protons that will probably contribute up to 3 Hz to the linewidth (see SCS). These two contributions would account fully for the observed 6 Hz. A covalently linked proton on the phosphoserine at a distance of 1.7 Å (in 50% D_2O) would give rise to an extra 7.7 Hz, so that the total theoretically calculated linewidth would then be almost 14 Hz, twice that experimentally determined. Moreover, since the observed phosphoserine resonance is probably indicative of two conformations (see above), the experimentally measured value of 6 Hz is an upper limit. From this result and the results of pH titration studies on several protein bound phosphoserines (Chapters 8,9,11), we conclude that the phosphoserine is in the dianionic form. This conclusion is also consistent with the complexation by arginine residues detected by X-ray crystallography.

Powder patterns of model compounds. As can be seen from a table of anisotropy factors (Brauer and Sykes, 1981, and references therein) the average values observed for the anisotropy term $\Delta\sigma(1 + \eta^2/3)^{1/2}$ for phosphomonoesters (like phosphoserine) are 120 ppm and those for phosphodiesters are about 170 ppm. Clearly, the values that we calculated for the two phosphohistidines (230 ppm) are larger. However, no results have yet been published for phosphoramidate and acyl-phosphate compounds. X-ray crystallographic studies have shown (Beard and Lenhert, 1968) that such compounds deviate from a tetrahedral structure. Hence large anisotropies can be expected. Table 2 shows, however, that the values we determined from powder spectra for imidazole-diphosphate, creatine-phosphate, and acetyl-phosphate are quite similar to those observed for phosphodiester compounds. The somewhat larger value found for 3'5' cAMP compared to other phosphodiesters is presumably caused by the fact that the phosphoryl residue is part of a six membered ring system. The value determined for phosphoserine is close to those for other phosphomonoesters. However, the values observed for the phosphoramidates clearly do not explain the larger anisotropies found for both protein-bound residues. Similarly, even larger values were reported for ATP bound to actin and phosphoglycerate kinase (Brauer and Sykes, 1981). Possible reasons for this anomaly are considered below:

1. The simplest explanation is that the estimated τ_c's used for these calculations are too short. We have assumed the proteins to be perfect spheres; deviations from that shape will lead to longer τ_c and hence to smaller anisotropy terms. In fact, when we subtituted the anisotropy value 187 ppm (determined from powder spectra of model compounds, Table 2) the τ_c's determined for SCS and HPr were 84 and 11 nsec, respectively. These values are larger than those calculated theoretically (Table 1) and compare favorably to τ_c's determined experimentally by ''F-NMR for alkaline phosphatase (Hull and Sykes, 1975) and by fluorescence depolarization measurements for a variety of proteins (Yguerabide *et al.*, 1970). It should be borne in mind, however, that the anisotropy value used to calculate these

Table 4.2

Values of the ^{31}P chemical shielding tensors of model compounds

Sample	σ_{11}	σ_{22}	σ_{33}	$\Delta\sigma(1+\eta^2/_3)^{1/2}$ [2]	Ref. [1]
Imidazole diphosphate (Ca)	125	-55	-70	187	1
Creatine phosphate (Na_2)	130	-20	-70	180	1
Acetyl phosphate (Li, K)	125	-30	-55	168	1
3',5'-cyclic AMP (acid)	125	55	-120	219	1
Phosphoserine (acid)	75	20	-50	110	1
3',5'-cyclic AMP	88	38	-124	192	2
Phosphoserine	46	3	-52	86	3

All values are in ppm relative to 85% H_3PO_4.
Downfield shifts are given a positive sign.
The values are accurate to ± 5 ppm.

[1] References are: (1) this report;
(2) Terao *et al.*, 1977;
(3) Kohler and Klein, 1976.

[2] Calculated with conventions described in Hull and Sykes (1977). These values are probably overestimated by *ca*. 15%. (See Materials and Methods).

τ_c's is possibly 15% overestimated when compared to literature values (see Table 2). Using a 15% lower anisotropy value, the calculated τ_c's for SCS and HPr would far exceed the experimental values. We are therefore inclined to the view that uncertainties in the value of τ_c cannot fully account for the larger anisotropy values determined.

2. As was discussed for phosphorylase *a*, the possibility of exchange between two different bound environments within the intermediate to fast exchange limit cannot always be excluded. This situation can lead to larger observed linewidths. The field-dependent contribution is a valid measure of the CSA contribution only when the resonance is that of a single conformation.

3. It has been proposed that part of the catalytic mechanism of *E. coli* Alkaline Phosphatase involves the induction of an unusual strain in the bond angles of the phosphoserine intermediate (Bock and Sheard, 1975; Chlebowski *et al.*, 1976). Thus another possible explanation is that binding to the protein could change the bond geometry of the substituents on the phosphorus atom which in turn could lead to the larger anisotropy values observed. This is known to cause changes in the chemical shift (Gorenstein, 1975). In fact, the larger CSA value found for the "strained" phosphodiester 3'5'-cAMP seems to support this view. It is then tempting to speculate that part of the catalytic power of phosphoryl transfer enzymes could involve

distortion of the phosphoryl group to a less tetrahedral configuration, thus facilitating the in-line nucleophilic attack that generally occurs (Knowles, 1980).

4. Changes in electronegativity of the phosphorus substituents caused by the binding to the protein molecule and related changes in the relative amounts of -bonding could lead to the larger anisotropy factors observed, since both can effect electron distribution around the phosphorus atom. These changes could also make the phosphorus atom more electrophilic, thus facilitating nucleophilic attack.

Conclusions

The results presented here for three different phosphoproteins with a range of molecular weights indicate clearly that at high field strength chemical shift anisotropy dominates the relaxation rates. Since the T_1 (spin lattice relaxation time) for this relaxation mechanism is field-independent (Shindo, 1980; Brauer and Sykes, 1981), the resolution and the signal to noise ratio will not improve but instead will decrease at higher magnetic fields (see Fig. 1). At low magnetic field strength, we find that the relaxation is largely determined by proton-phosphorus dipole-dipole interactions, in agreement with earlier suggestions (Ray *et al.*, 1977). However, Figs. 2 and 3 show that even for frequencies as low as 36.4 MHz a considerable portion of the relaxation is caused by field-dependent terms

(25 and 55% respectively). This casts doubt on the validity of calculations that assume domination by either one of the mechanisms at these low frequencies. In contrast, as the frequency is increased, contributions due to dipole-dipole relaxation reduce to less than 10% at 162 MHz.

From a comparison of the data for the N-3 phosphohistidine of *E. coli* succinyl-CoA synthetase and the phosphoserine on rabbit muscle glycogen phosphorylase we conclude that they are in their monoanion and dianion forms, respectively. This conclusion for the phosphohistidine residue supports the suggestion by Gassner *et al*. (1977) who propose a pKa = 8-9, over that of Hultquist *et al*. (1966) who suggest a much lower pKa. The cooperative inhibition of succinyl-CoA synthetase by NO_3^- but not by other double negatively charged anions supports this view (see Chapter 6). se studies provide for analysis of linewidth in terms of functional biochemical properties of three limiting cases of phosphoproteins. These are systems with immobile phosphoryl substituents, those with mobile phosphoryls, and those characterized by more than one conformation:

1. It is of particular interest that for both active site phosphohistidines the phosphorus atom appears to be virtually immobilized on the protein. A similar result was reported for the phosphoglucomutase active site phosphoserine residue (Ray *et al*., 1979), and the linewidth reported for metal-saturated alkaline phosphatase (20 Hz at

40.5 MHz, Hull *et al.*, 1976) also implies an immobile residue. T_1 measurements by Chlebowski *et al.* (1976) have suggested some flexibility for this residue in the apoenzyme, but these workers also reported restriction of motion in metal-saturated enzyme. All of this suggests that immobility of the phosphorylated amino acid residue may be a general property of catalytic intermediates of phosphoryl-transferring enzymes.

2. In contrast, studies on regulatory phosphoserine residues have indicated significant mobility. Not only was this observed for the regulatory serine phosphate of phosphorylase *a* as outlined here, but this was also demonstrated for similar sites on tropomyosin (Chapter 8), ovalbumin (Chapter 9), troponin T (Sperling *et al.* 1979), and myosin light chains (Koppitz *et al.* 1980). This mobility may be required to facilitate access to the regulatory site by specific protein kinases and phosphatases.

3. Resonances with larger linewidth than those deduced from our calculations here have been seen, for example, for the pyridoxal-phosphate (form I) of glycogen phosphorylase after addition of the competitive inhibitor α-D-glucopyranosyl cyclic-1,2-phosphate (Withers *et al.*, 1981) and for the broadening of the phosphohistidine resonance of succinyl-CoA synthetase in the presence of CoA (Vogel and Bridger, 1981). This extra broadening must be attributed to exchange between at least two conformations with different

chemical shifts.

The results obtained here should thus have general applicability to similar studies on phosphoproteins or enzymes with covalently linked coenzymes.

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CHAPTER 5

A ^{31}P-NMR STUDY OF THE INTERMEDIATES OF THE *ESCHERICHIA COLI* SUCCINYL-COENZYME A SYNTHETASE REACTION.[1]

Evidence for substrate synergism and catalytic cooperativity

Summary

Escherichia coli succinyl-CoA synthetase has an $\alpha_2\beta_2$ structure (mol. wt. 140,000) with the two active sites arranged at the interface of the α and β subunits. Here we describe ^{31}P-NMR experiments confirming the existence of two phosphorylated intermediates in catalysis. The phosphohistidyl resonance is readily observed at -4.8 ppm. This resonance is shifted upfield in the presence of Mg^{2+} and broadened by Mn^{2+}, indicating interaction between the metal ion and the phosphoryl group. The addition of succinate alone is without effect on the spectrum. The presence of CoA causes a downfield shift and broadening of the phosphohistidyl resonance to a linewidth sufficiently large to be indicative of two exchanging conformations. The presence of both CoA and the competitive inhibitor 2,2-difluorosuccinate seems to freeze the phosphoryl group in one orientation. The addition of ATP to this already phosphorylated enzyme leads

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[1] Preliminary accounts of this work were presented at the 14th FEBS Meeting in Edinburgh, Scotland (1981) and the 24th Meeting of the Canadian Federation of Biological Societies, Montreal, Canada (1981).

to appearance of a succinyl-phosphate resonance. This last result is in harmony with the concept of alternating sites catalytic cooperativity previously proposed for this enzyme, since it implies that ATP binding or phosphorylation of histidine at one active site triggers phosphoryl transfer from histidine to succinate at the other site. If the non-hydrolyzable β,γ-methlylene analogue of ATP is used, however, this produces no change in the phosphohistidyl resonance. This suggests that intrasubunit communication is triggered by phosphorylation by ATP rather than by binding of nucleotide. We also observed CoA-induced changes in the spectrum of the enzyme-bound β,γ-methylene analogue of ATP, consistent with the previously observed CoA mediated enhancement of the ATP $\rightleftharpoons$ ADP exchange which is known as "substrate synergism". A detailed model for catalysis consistent with these observations is presented.

Introduction

Succinyl-CoA synthetase (SCS) from *E. coli* is a tetrameric enzyme with an $\alpha_2\beta_2$ structure and an overall molecular weight of 140,000 (1,2). The enzyme is best described as a dimer of dimers, since it has been shown that it contains two active sites each arranged at the interface of an α and β subunit (3-5). The reaction catalyzed is believed to proceed via three partial reactions with a covalent N-3 his-*P* residue and an enzyme-bound succ-*P* (6) participating as discrete intermediates:

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{E-P} + \text{ADP} \tag{1}$$

$$\text{E-P} + \text{succinate} \rightleftharpoons \text{E}\cdot\text{succ-}P \tag{2}$$

$$\text{E}\cdot\text{succ-}P + \text{CoA} \rightleftharpoons \text{E} + \text{succinyl-CoA} + \text{Pi} \tag{3}$$

$$\text{ATP} + \text{succinate} + \text{CoA} \rightleftharpoons \text{succinyl-CoA} + \text{ADP} + \text{Pi}$$

It has been shown that the phosphorylated enzyme satisfies the kinetic criteria required of an obligatory intermediate in catalysis (7). The catalytic competence of the succ-*P* intermediate (8) has been a subject of some controversy (9), which was settled principally by the demonstration that the rates of succ-*P* utilisation and production were comparable to the overall rates in the presence of the analogue desulfo-CoA (10,11). This observation is one of the many demonstrations of a property of the enzyme known as substrate synergism, viz., the enzyme is fully active only when all substrate binding sites are occupied (7).

Another property of the enyzme that is pertinent to the subject of this paper is the apparent half-of-the-sites phosphorylation by ATP; only one of the two α subunits is readily phosphorylated (12,13). Similar effects have recently been reported for malate thiokinase of *Pseudomonas* MA, which has a similar subunit structure and catalyzes a virtually identical reaction (14,15). Since enzymes showing negative cooperativity or half-of-the-sites reactivity are likely candidates for alternating sites cooperativity, Bild *et al.* (16) investigated the enzyme for this property. They demonstrated an ATP modulation of the extent of oxygen exchange between medium [^{18}O]Pi and [^{18}O] succinate for the tetrameric *E. coli* enzyme. No modulation was detected for the dimeric pig heart enzyme, leading them to propose that the binding of ATP to one active site promotes catalytic events at the other (*i.e.*, catalytic cooperativity) and that the two active sites work in an alternating fashion (*i.e.*, alternating sites cooperativity). Capacity for alternating sites cooperativity has been confirmed for the *E. coli* enzyme (17).

In this communication we report ^{31}P-NMR studies of the two catalytic intermediates. While ^{31}P-NMR has provided useful information about catalytic involvement of the active site phosphoserine residue of *E. coli* alkaline phosphatase (18,19) and the pyridoxal phosphate residue of rabbit muscle glycogen phosphorylase (20,21), NMR studies on his-*P*-containing proteins to date have been primarily confined to determination of the position of phosphorylation of the histidyl residue (22-24). Since the enzyme has the property of substrate synergism and possibly that of alternating sites cooperativity, we have concentrated on the effects of the addition of substrates on the ^{31}P-resonances observed for the N-3-his-*P* and succ-*P* respectively. Further evidence for substrate synergism and catalytic cooperativity has been obtained.

Experimental Procedures

Succinyl-CoA synthetase was purified from Crooke's strain of *E. coli* grown on succinate-based medium by methods described earlier (25,26). Preparations were judged to be pure by homogeneity upon gel electrophoresis. Assays for activity were performed as described by Ramaley *et al.* (12). The concentration of the enzyme was determined from its absorbance at 280 nm (2). Samples for NMR experiments containing 1.0 phosphoryl groups per $\alpha_2\beta_2$ tetramer were prepared as described earlier (27-29) except that Millipore molecular separators were used instead of ultrafiltration to concentrate the samples. It has been shown that this procedure efficiently eliminates paramagnetic impurities (28,29). The specific activity was measured before and after obtaining ^{31}P-NMR spectra and was never below 32 units/mg enzyme. All NMR samples were in a buffer containing 40 mM Tris-HCl, 80 mM KCl, 20% D_2O, pH 7.2.

Coenzyme A and adenosine 3'5' diphosphate were obtained from P.L. Biochemicals, ATP was from Terochem (Edmonton, Canada), 2,2-difluorosucccinate was purchased from Sigma, Tris (ultra pure) from Schwarz-Mann and D_2O from Biorad. Other chemicals were analytical grade. Phosphohistidine was synthesized as described by Rosenberg (30). Succ-*P* was prepared by mixing succinic anhydride (in ethanol) in a phosphate buffer (31). The ^{31}P-NMR chemical shifts measured for these preparations are comparable to those seen for acetyl-phosphate (Sigma). ^{31}P-NMR spectra were obtained as described earlier (32,33) at a frequency of 109.3 MHz. Samples (1.5 ml) were placed in 10 mm precision tubes (Wilmad) equipped with Teflon vortex plugs. The sample temperature was kept constant at 27±1°C. All spectra are plotted with 25 Hz additional linewidth by means of computer digital filtering, unless otherwise indicated.

Results

Effects of succinate and Mg^{2+} on his-P residue. Since the γ- and β-phosphoryl residues of ATP and ADP respectively in the presence of Mg^{2+} give rise to a resonance in the ^{31}P-NMR spectrum with similar chemical shift to those of his-*P* standards (22), we could not study phosphorylation of the enzyme (partial reaction 1) directly. Therefore all subsequent experiments were performed with enzyme that was phosphorylated with ATP and isolated by gel filtration, according to the procedure of Wang *et al*. (27). The product is a preparation containing one phosphoryl group per $\alpha_2\beta_2$ tetramer.[2] A typical ^{31}P-NMR spectrum of E-*P* is shown in Fig. 1A. The chemical shift position (-4.8 ppm) is characteristic of an N-3 his-*P* residue (22). The frequency dependence of the linewidth, reported elsewhere (32), has led us to conclude that the residue is rigidly held to the

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[2] The degree to which the enzyme can be phosphorylated has been controversial (1,5,12,13,34). We have recently obtained evidence that this may be related to dissociation of the tetramer to α,β dimers at low concentration (M.D. O'Connor and W.A. Bridger, in preparation).

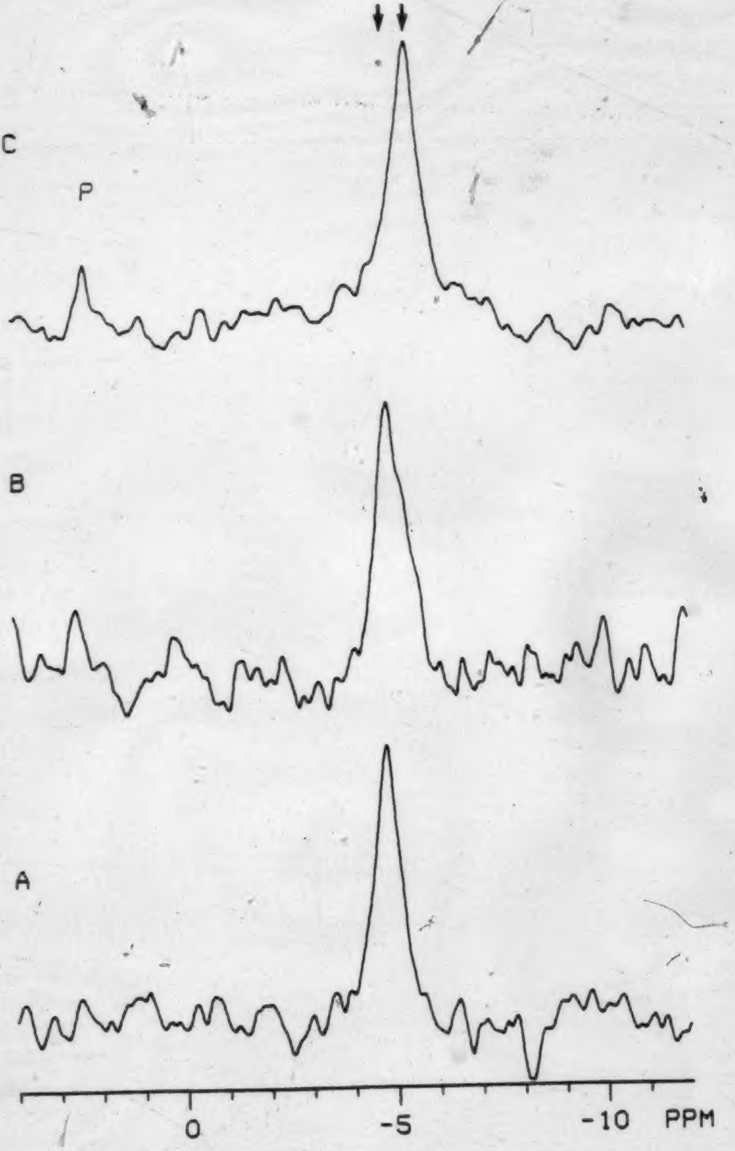

Fig. 5.1 ^{31}P-NMR spectra at 109.3 MHz of *E. coli* succinyl-CoA synthetase (SCS). (A) 24 mg/ml of enzyme, 7500 scans; (B) 5 mM succinate and 24 mg/ml of SCS, 6000 scans; (C) 2.5 mM $MgCl_2$ and 24 mg/ml of SCS, 10000 scans. Other conditions as described in Experimental Procedures.

enzyme and that it is in a monoanion form.

Figure 1B shows the spectrum of the same sample after addition of 5 mM succinate. Clearly no changes in linewidth or chemical shift position occur. Even with concentrations of succinate up to 25 mM only minor upfield shifts (<0.2 ppm) were observed. Although the succinate-binding site should be saturated under these conditions, the his-*P* seems hardly affected by its presence. The requirement for a metal ion for the partial reactions can be fulfilled by either Mg^{2+} or Mn^{2+} (35). Proton relaxation rate enhancement studies have revealed four Mn^{2+} binding sites with similar affinity for the phosphorylated and dephosphorylated enzyme (28,29). Thus, it is not clear whether the phosphoryl group is directly involved in the binding of the metal ion. Fig. 1C shows that addition of 2.5 mM $MgCl_2$ leads to an upfield shift of 0.6 ppm (-4.8 to -5.4 ppm) as indicated by the arrows. Titration studies with Mg^{2+} showed an increase in the his-*P* chemical shift with concentration and that high Mg^{2+} concentration induces loss of the phosphoryl moiety. Reciprocal plots of change in chemical shift versus [Mg^{2+}] suggested a Kd near 4 mM. This value agrees reasonably with the value of 10 mM obtained (35) for maximal activity of the enzyme. Since lower concentrations of Mn^{2+} are necesary for maximal activity, this presumably also corresponds to the Kd of 0.7 mM determined for Mn^{2+} binding to the phosphorylated enzyme (28). The results of the Mg^{2+} titrations were similar in the presence and absence of succinate.

Since Mg^{2+} effects on the enzyme's his-*P* residue are not sufficient sole evidence for direct interaction between the phosphoryl group and the metal ion, we studied the effect of addition of paramagnetic Mn^{2+} ions. This caused broadening of the residue beyond detection, which was reversible by the addition of EDTA. This places the metal ion within 10 Å of the phosphoryl group, suggesting that it interacts with it but is not necessarily coordinated to it. Therefore we propose that the binding site for the metal ion is mainly supplied by the enzyme (29) and a weak interaction exists with the monanionic his-*P* residue. Such a mode of binding would explain the small difference in the Kd's found for Mn^{2+} binding to phosphorylated and dephosphorylated enzyme (0.74 mM and 1.4 mM respectively) (28). In keeping with proposals made for alkaline phosphatase (18,19), the chemical shift changes caused by Mg^{2+} binding could then be explained by a change in bond geometry at the phosphoryl group.

Effects of CoA binding. Figs. 2A and 2B demonstrate the effects of simultaneous succinate and Mg^{2+} addition to phosphorylated enzyme. The Mg^{2+}-induced upfield shift (-4.8 to -5.6 ppm)is again observed. Although all reactants necesary for partial reaction 2 are present (E-*P*, succinate and Mg^{2+}), no reaction occurs. Apparently this step requires the interaction of the other substrates with their binding

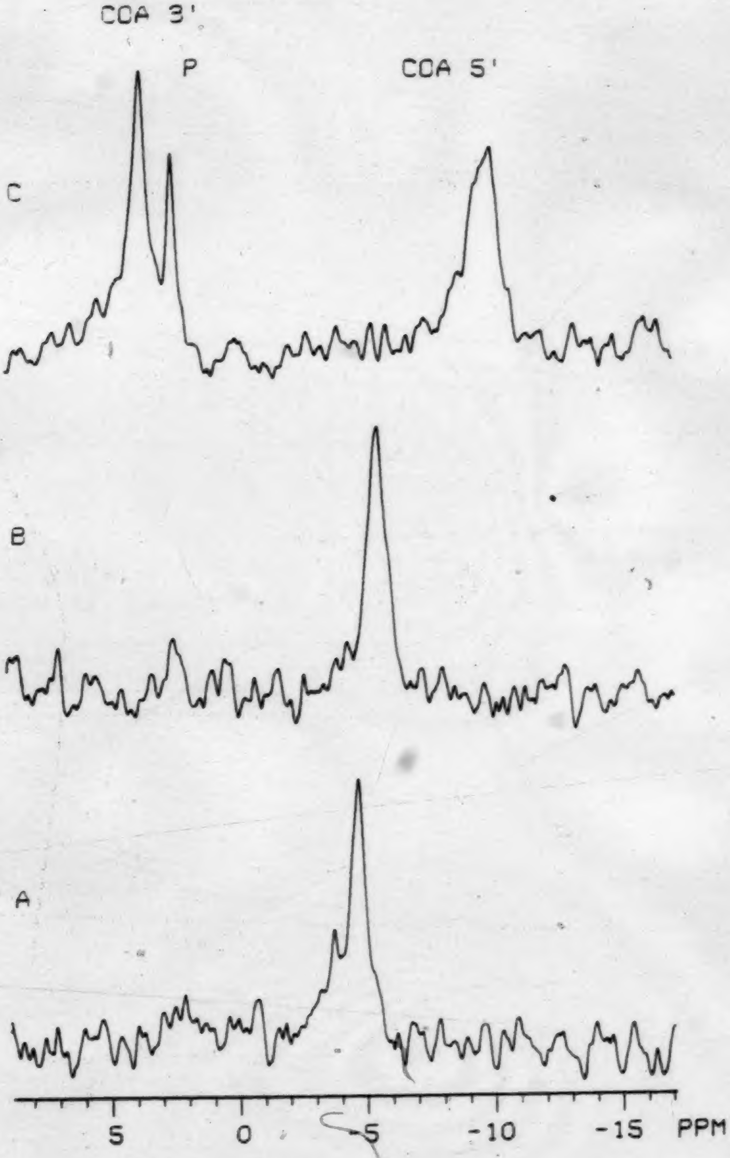

Fig. 5.2 ^{31}P-NMR spectra of 28 mg/ml SCS, with the following additions: (A) no additions, 7500 scans; (B) 10 mM succinate, 4 mM $MgCl_2$, 12500 scans; (C) as in B, plus 0.35 mM CoA, 12500 scans. Other conditions as described in Experimental Procedures.

sites, consistent with the concept of substrate synergism previously reported for isotope exchange reactions (7).

Fig. 2C shows that after addition of CoA, the his-*P* resonance at -5.6 ppm has disappeared and has given rise to a resonance at 2.4 ppm which we have identified as inorganic phosphate. The most upfield and downfield (-10 and 3.6 ppm) peaks can be assigned to the 5'-pyrophosphoryl and 3'-phosphoryl moieties of CoA. The linewidth of the resonances observed for these residues are indicative of binding of the coenzyme to the enzyme since they exceed those measured for a sample of CoA in the absence of enzyme. The low Kd for CoA (1.5 μM) (1) would suggest slow exchange between free and bound coenzyme. However, no separate peaks have been observed; perhaps the chemical shifts for the bound and free form are identical. Unfortunately, several trials have not allowed clear definition of the mode of binding. The binding of CoA is very sensitive towards small changes in the specific activity of the enzyme (34), and this was further substantiated by our spectral data. Fig. 2C shows clearly that partial reaction 2 can occur in the presence of CoA. Since no succ-*P* line is observed one might infer that succ-*P* is not a true intermediate and that partial reaction 2 and 3 occur in concerted fashion. However, evidence is presented below that strongly supports the intermediate formation of succ-*P*, indicating that partial reactions 2 and 3 are both fast relative to the time course of the experiment. Clearly, this is another demonstration of substrate synergism, namely that the presence of CoA enhances the rate of partial reaction 2. This is similar to the earlier demonstration of the effect of the analogue desulfo-CoA on the rate of succ-*P* production and utilization (10,11).

The means by which CoA stimulates partial reaction 2 is further examined by the series of spectra shown in Figs. 3 and 4. Comparison of Figs. 3A and 3B shows that CoA addition results in a downfield shift from -4.8 to -4.1 ppm, together with broadening of the 55 Hz-wide his-*P* resonance. In a separate communication (32), we have reported the frequency dependence of the linewidth for the his-*P* residue. The his-*P* residue is immobilized on the protein surface, and thus the observed linewidth of 55 Hz is an upper limit. Resonances with linewidths exceeding this figure, as we see here for the his-*P* residue after addition of CoA, must therefore originate from exchange broadening, and are taken as evidence for the existence of not less than two exchanging conformations (32). The other resonances in spectrum 3B originate from CoA (-10.1 and 3.7 ppm) and from Pi at 2.1 ppm. The appearance of Pi here is consistent with the report (11) that CoA can induce an ATPase activity in the enzyme. Apparently, the his-*P* is most stable in its conformation signalled by a chemical shift of -4.8 ppm. Both the Mg^{2+}-induced upfield shift and the CoA induced broadening and downfield shift lead to loss of enzyme his-*P* to solution

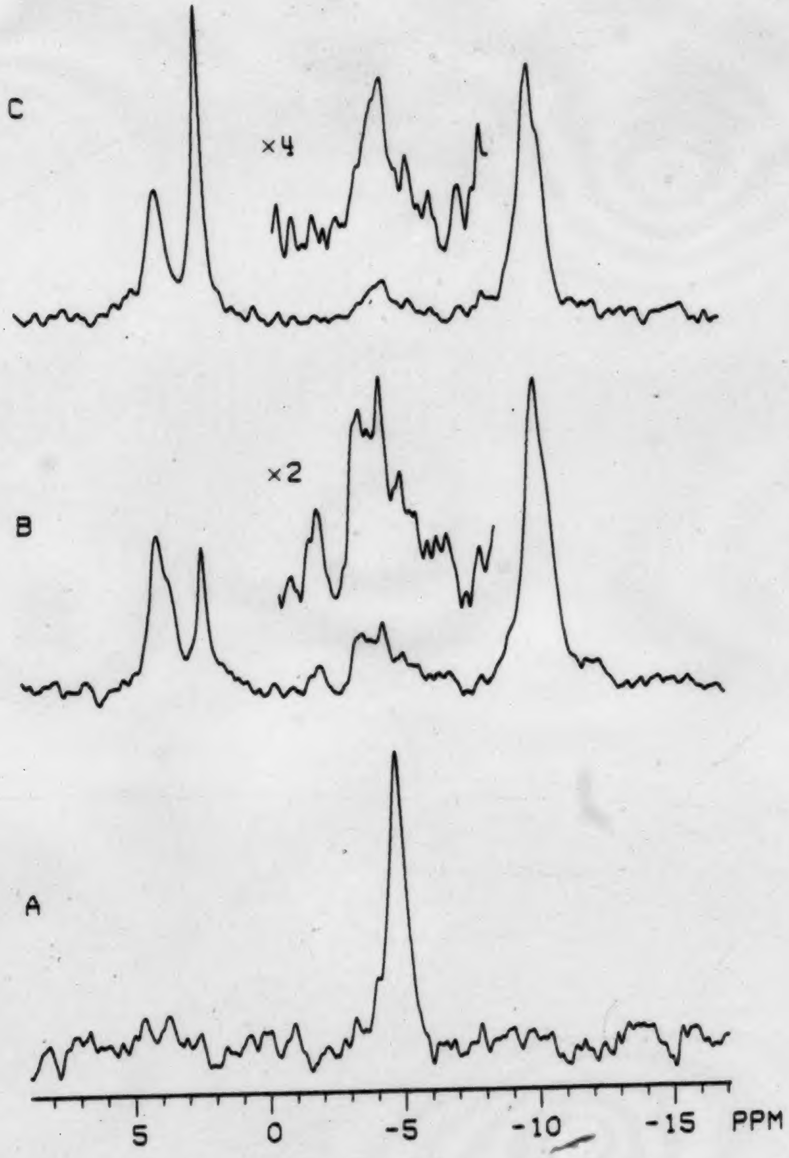

Fig. 5.3 ³¹P-NMR spectra of 19.0 mg/ml SCS with the following additions: (A) No additions, 27500 scans; (B) 2 mM dithiothreitiol, 0.50 mM CoA, 20000 scans (dithiothreitiol alone has no effect on spectrum); (C) as in B, plus 5 mM 2',2 difluorosuccinate, 20000 scans. Other conditions as described in Experimental Procedures. For assignment of peaks see text and Figure 2.

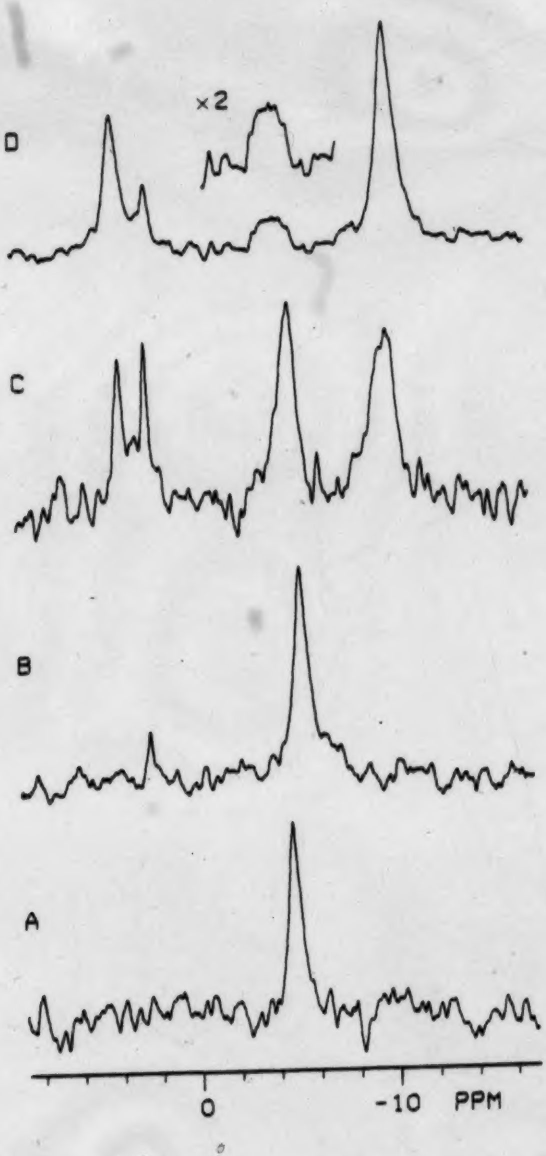

Fig. 5.4 ^{31}P-NMR spectra of 26 mg/ml SCS with the following additions: (A) No additions, 7000 scans; (B) 2.5 mM $MgCl_2$ plus 2 mM dithiothreitol, 12500 scans; (C) as in B, plus 0.12 mM CoA, 10000 scans; (D) as in C, but CoA concentration increased to 0.60 mM, 8500 scans. Other conditions are described in Experimental Procedures. The peak assignments are similar to those of Figs. 2 and 3.

inorganic phosphate.

A titration study of the broadening effect, this time in the presence of Mg^{2+} and 2.5 mM dithiothreitol, showed that at half saturation (one CoA per tetrameric enzyme molecule, Fig. 4C) the effect is largely manifested as a downfield shift (-5.4 to -5.1 ppm) with almost a doubling of the linewidth to 100 Hz. (These shifts are higher than the usual -4.8 ppm because of the presence of 2.5 mM $MgCl_2$: see Figs. 4A and 4B. It is noteworthy that the CoA-induced downfield shifts and the Mg^{2+}-induced upfield shifts are additive.) Subsequent saturation of the total enzyme molecule shows a further downfield shift to -4.5 ppm and more severe broadening (see Fig 4D).[3] Without knowledge of the order of CoA binding to the two active sites (of which one is phosphorylated) it is not possible to distinguish if these effects are caused by binding to the phosphorylated or to the other active site. Since saturation of the enzyme is required for the full effect on the his-*P*, it seems that binding to both active sites is a necessity for the observed changes. Therefore, the phenomena previously attributed to substrate synergism (*i.e.*, cooperativity within one active site), could as well be caused by catalytic cooperativity (*i.e.*, cooperativity between events in two active sites).

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[3] Since the enzyme has only two binding sites for CoA (34), binding of CoA to regulatory sites can be ruled out as a possible explanation for these phenomena.

The addition of CoA in either the presence or absence of Mg^{2+} gave rise to extensive broadening and downfield shifts in six separate experiments but no consistent lineshape could be found (compare Figs. 3B, 4D and 6C). It should be borne in mind that the differences in CoA binding and the CoA-induced ATPase activity complicate such measurements; similar problems have been encountered with other enzymes (36).

In a subsequent experiment, instead of adding succinate and $MgCl_2$ (which would lead to the result of Fig. 2C) we added the competitive inhibitor 2,2-difluorosuccinate (Fig. 3C). The broadened resonance clearly sharpens to a resonance with a linewidth indicative of only one conformation (32), and shifts back upfield to -4.65 ppm. Apparently, the phosphoryl group is now locked into one position, perhaps ready for transfer if the correct substrate (succinate) were in place. Although the chemical shift position of this resonance is virtually indistinguishable from that observed for phosphorylated enzyme in the absence of substrates, this does not necessarily imply the same conformation (33).

When the additions in the experiment of Fig. 3 were made in the opposite order (by adding fluorosuccinate first and CoA second) the identical end result was obtained, indicating that the order of addition is not important.

Because of the effects of desulfo-CoA on succ-*P* production and utilisation (10,11) it was of interest to determine whether CoA analogues could give a similar broadening, since this would directly relate the observed broadening to the enhancement of catalytic events. The presence of 1 mM adenosine-3',5'-diphosphate (*i.e.*, CoA without the pantothenoic acid residue) did not cause any broadening or change in chemical shift. Consistent with this is that no effect on the rate of succ-*P* production could be demonstrated (data not shown). Similarly the analogue oxy-CoA did not exert effects on this partial reaction (10). Thus these observations are consistent with the proposal that the CoA-induced broadening signals the presence of two exchanging conformations for the his-*P*, and that exchange between these two conformations is necesary for catalysis.

Effects of ATP on Phosphohistidine Residue. The concept of catalytic cooperativity would imply that ATP binding to one active site promotes catalytic events at the other (16). The binding of Mg-ATP to phosphorylated enzyme is known to be very weak (28,34). Figure 5 shows again that partial reaction 2 does not occur independently (Fig. 5A, 5B). When ATP is added (Fig. 5C) it is clear that the his-*P* resonace is obscured by the ATPγ (-4.9 ppm) and ADPβ (-5.6 ppm) resonances. However, two new resonances appear in the downfield region; the one at 2.2 ppm is Pi and the one at -1.5 ppm we have assigned to succ-*P*. The narrowness of the

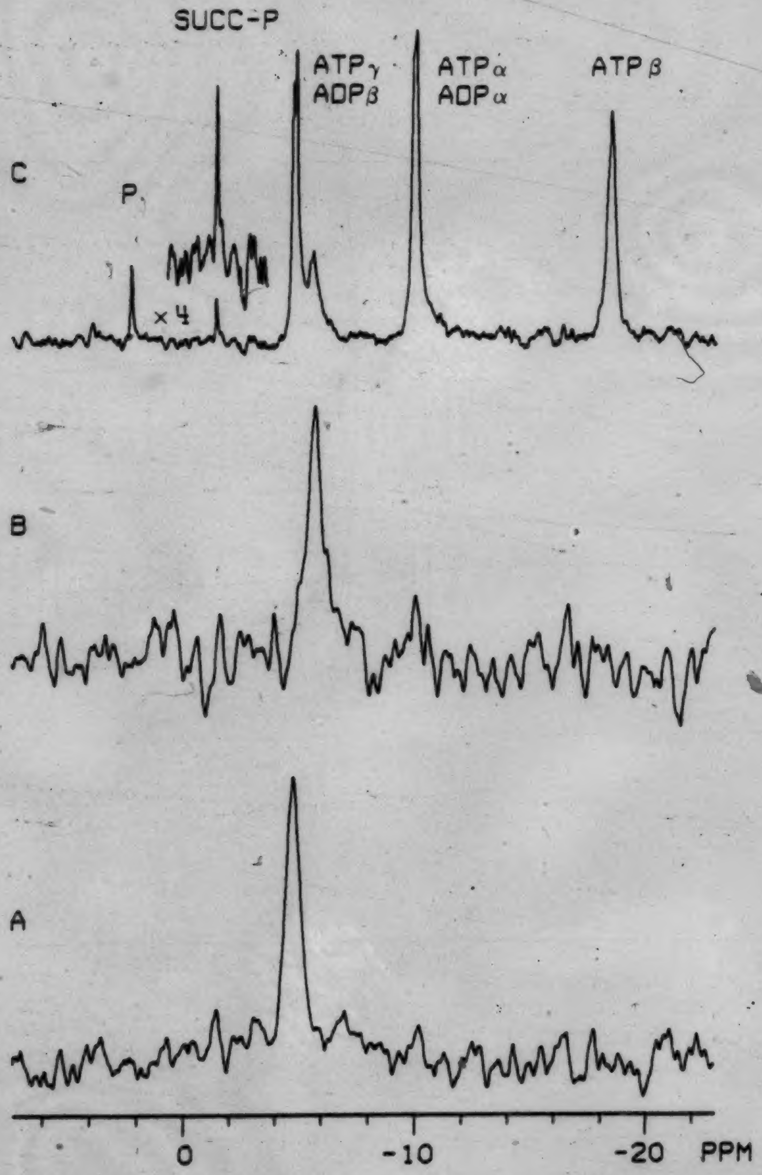

Fig. 5.5 ^{31}P-NMR spectra of 22 mg/ml SCS with the following additions: (A) no additions, 27500 scans; (B) 5 mM succinate and 4 mM $MgCl_2$, 12500 scans; (C) as in B, plus 2.5 mM ATP, 2500 scans. Other conditions are as described in Experimental Procedures.

succ-*P* line indicates that if this compound is bound to the enzyme it is not rigidly held. It is also possible that this noncovalently bound intermediate is released from the enzyme; since it is not very stable in solution (31), it would hydrolyse to succinate and P . This would account for the appearance of the Pi resonance in the spectrum. In another experiment where the time course of this reaction was followed, the ATP resonance declined, the succ-*P* passed through a maximum, and only the Pi and ADP resonances kept increasing (data not shown), in keeping with the idea that succ-*P* is released from the enzyme and subsequently hydrolyzed in solution. Since these data indicate that the affinity of the enzyme for this intermediate is low, it is remarkable that during normal catalysis this intermediate is not detectable in solution. Apparently partial reaction 3 must be fast, consistent with our interpretation of Fig. 2C. In fact, it has been demonstrated that it proceeds nonenzymatically at considerable rate in solution (37).

Effects of binding of AMPPCP. The results presented in Fig. 5 give strong support to the proposal that ATP binding to one active site (nonphosphorylated) triggers catalytic events at the other (phosphorylated) site (16). Since use of ATP does not allow differentiation between effects caused by binding or phosphorylation by the nucleotide, we approached this question by the use of nonhydrolyzable ATP analogues. The Mg^{2+} complex of β,γ-imido ATP could not be used since its β resonance would overlap that of his-*P*. The β,γ-methylene ATP analogue is a competitive inhibitor (Ki =0.38 mM), has more advantageous chemical shifts, and in the presence of Mg^{2+} bears close resemblance to ATP (33). Figs. 6A and 6B clearly indicate that the presence of saturating amounts of this analogue does not bring about changes in the phosphohistidyl residue. The peaks at 15.0, 9.9 and -10.3 ppm are assigned respectively to the γ, β and α-phosphorus atoms of the analogue. Upon addition of Mg^{2+} (Fig. 6C), large changes in the analogue spectrum are observed (33), but the his-*P* shows its normal Mg^{2+}-induced upfield shift (-4.8 to -5.2 ppm). Fig. 6D shows that addition of succinate to this mixture does not lead to a disappearance of the his-*P* residue. Although the acquisition time for spectrum 6D is seven times that of spectrum 5C, clearly no succ-*P* line is observed. Thus the analogue is unable to duplicate the action of ATP, indicating that phosphorylation by ATP rather than binding leads to succ-*P* production.[4]

Effects of CoA on AMPPCP binding. To further substantiate that binding of the ATP analogue AMPPCP does not lead to changes in the properties of the enzyme, we performed the experiments depicted in Fig. 7. The results of Figs. 7A and

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[4] Since phosphorylation of the enzyme, rather than ATP binding, appears to be responsible for intrasubunit communication, simple binding of ATP to a possible regulatory site cannot account for the reported oxygen exchange patterns (16).

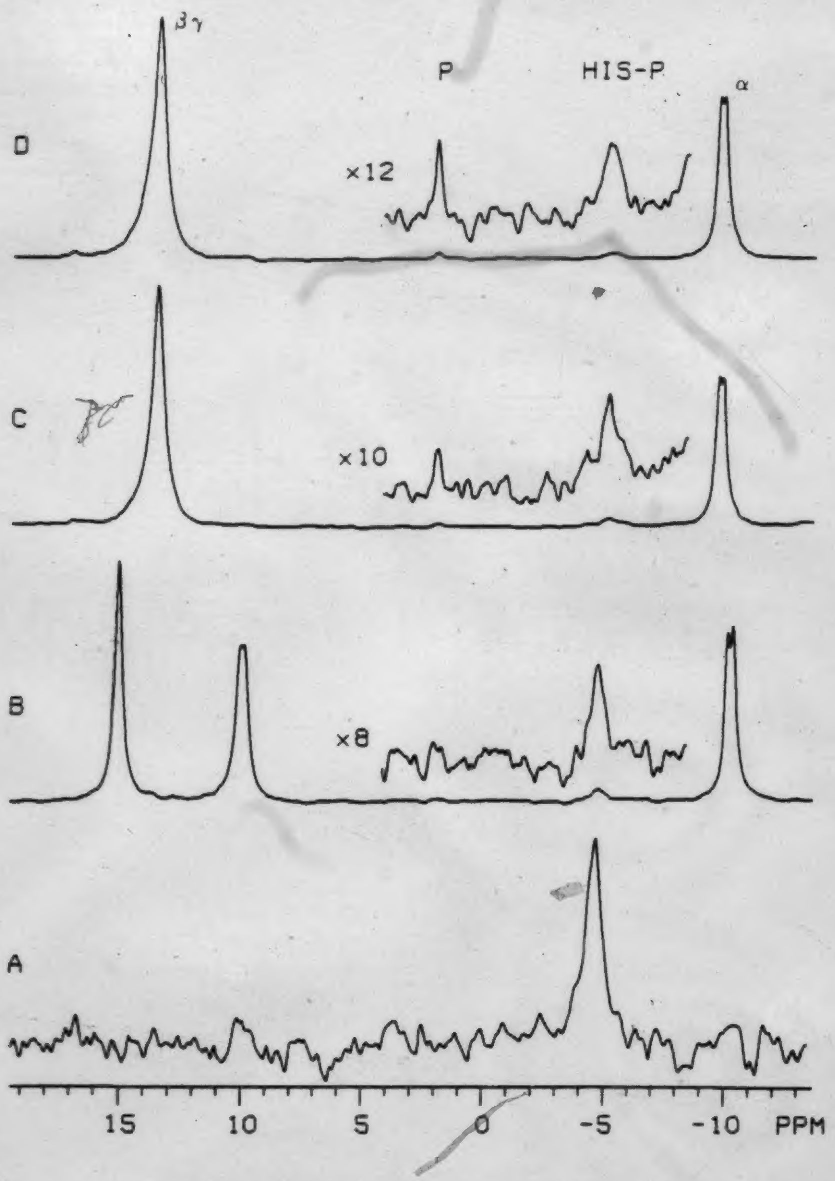

Fig. 5.6 ^{31}P-NMR spectra of 22 mg/ml of SCS with the following additions: (A) no additions, 20000 scans; (B) 2.5 mM AMPPCP, 7500 scans; (C) as in B, plus 3.4 mM $MgCl_2$ 10000 scans; (D) as in C, plus 10 mM succinate, 17500 scans. Other conditions are as described in Experimental Procedures. α, β and γ refer to the assignment of the AMPPCP peaks. In spectrum 6B, the two most downfield peaks originate from the γ-P and β-P (left to right).

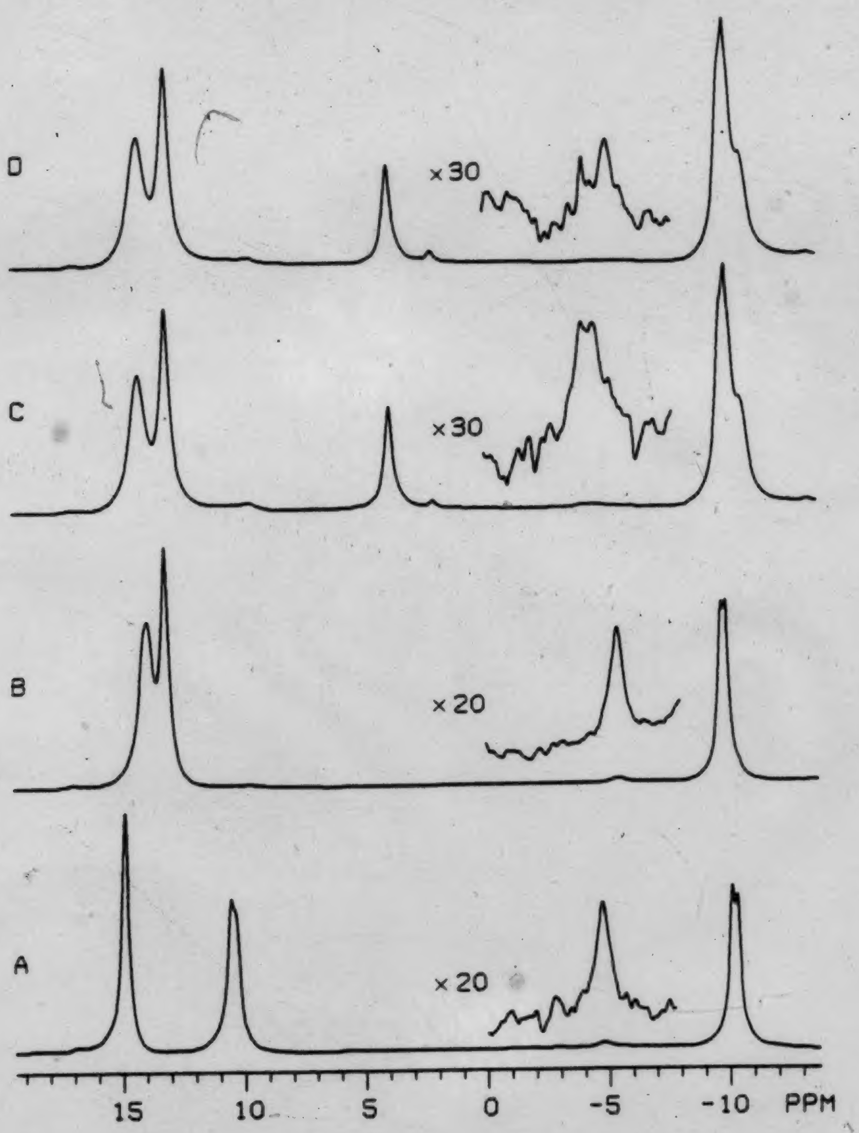

Fig. 5.7 ^{31}P-NMR spectra of 25 mg/ml SCS with the following additions: (A) 5 mM AMPPCP (see Fig. 6), 8000 scans; (B) as in A, plus 9 mM $MgCl_2$, 20000 scans; (C) as in B, plus 0.5 mM CoA, 15000 scans; (D) as in C, plus 5 mM 2',2-difluorosuccinate. Other conditions as described in Experimental Procedures. Assignment of peaks is shown in Figure 3 and is described in the text. Note that in Figure 7A AMPPCP γ-P is the most downfield resonance, while in Figures 7B, C, and D, AMPPCP β-P is the most downfield resonance. The pyrophosphate residue of CoA and the α-P of AMPPCP overlap in the most upfield peak of spectra 7C and 7D.

7B are consistent with an earlier observation (Fig. 6) that binding of AMPPCP or Mg-AMPPCP does not lead to any changes in the chemical shift position or linewidth of his-*P* (-4.8 ppm). The peaks in spectrum 7A, going from downfield to upfield, are assigned to the γ, β and α-P of the analogue, respectively. Addition of $MgCl_2$ causes expected changes in the AMPPCP spectrum. Note that the his-*P* has undergone its characteristic Mg^{2+}-induced upfield shift (-4.8 to -5.5 ppm).[a] Addition of CoA (spectrum 7C) leads to the characteristic downfield shift (to -4.3 ppm) and broadening of the phosphohistidyl residue. In spectra 7C and 7D the resonances at 3.8 and -10.6 ppm (overlapping with AMPPCPα) originate from CoA. Subsequent addition of 2,2-difluorosuccinate leads to an upfield shifted sharp resonance (-5.1 ppm) as seen earlier in the absence of the ATP-analogue. Thus, the presence of AMPPCP does not lead to changes in the measurable properties of the enzyme, in keeping with the observation that it does not induce succ-*P* production (Fig. 6).

Clearly, the β- and γ-P resonances (the two most downfield peaks) of AMPPCP are affected by the addition of CoA (compare Fig. 7B and 7C). This is further substantiated by the data of Table 1. Comparison of the chemical shifts measured for the ATP analogue in the presence and absence of enzyme but in the absence of CoA shows that only small

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[a] The binding of Mg^{2+} has resulted in a change of the positions of the β and γ resonances. This differs from Fig. 5C because of a change in the Mg^{2+}/nucleotide ratio. Full titration curves are described elsewhere (33).

Table 5.1

Measured chemical shifts (ppm) for AMPPCP at pH 7.2
(data see Fig. 5.7)

	γ	β	α	Jαβ (Hz)
NO ENZYME				
5 mM AMPPCP	14.75	10.45	-10.39	26
+ 9 mM $MgCl_2$	13.09	13.90	-9.96	23
WITH ENZYMES (25 mg/ml)				
5 mM AMPPCP	14.77	10.46	-10.38	26
+ 9 mM $MgCl_2$	13.10	13.82	-9.95	23
+ 0.5 mM CoA	13.04	14.16	-9.94	23
+ 10 mM F-succ.	13.04	14.17	-9.94	23

changes occur upon binding to the enzyme. Upon addition of CoA (in the presence and absence of 2,2-difluorosuccinate) a change in the β resonance of 0.3 ppm is observed. Assuming that fast exchange conditions prevail we can apply the following formula:

$$\delta_{obs} = \delta_{bound} \cdot f_{bound} + \delta_{free} \cdot f_{free}$$

with δ and f respectively the chemical shift and molefraction. Since the enzyme is phosphorylated at one active site it presumably has only one binding site for AMPPCP; therefore we calculate for the bound form that the β resonance is shifted downfield by 7.5 ppm and the γ resonance is shifted upfield by 1.7 ppm. (If one assumes two binding sites for ATP the shifts are 3.7 and 0.8 ppm respectively.) The chemical shifts calculated in this way clearly place the enzyme-bound form outside of the range of chemical shifts measured for solution structures (33). The directions of the shifts suggest that the bound form is in the deprotonated form with Mg^{2+} ion tightly complexed especially to the β phosphorus of the ATP analogue. This "tightening up" of the AMPPCP binding by CoA may be the basis for the earlier observed enhancement of the ADP$\leftrightarrow$ATP exchange reaction by CoA (7) which was the first demonstration of the substrate synergism for this enzyme.

The change in chemical shift calculated for the bound form of the ATP analogue (7.5 ppm) is large compared to those earlier reported for ATP binding to other kinases (36) and may be a reflection of the greater sensitivity of chemical shifts of methylene compounds towards changes in environment (33).

Discussion

The spectra presented here show for the first time that the his-*P* resonance on a protein of this size can be readily observed. The chemical shift of the phosphohistidyl resonance is very close to that of his-*P* in solution (22). In the case of alkaline phosphatase (18,19), a large downfield shift was reported for the active site phosphoserine residue which was attributed to bond strain. Similarly, studies on the bacterial heat-stable phosphoryl carrier protein (HPr) showed a downfield shift for the N1-phosphohistidine (22,23), whereas the resonance for one of the N-3-phosphohistidines of histone displays a large upfield shift (24). Our data, together with the normal shift of the phosphoserine residue of phosphoglucomutase (38), show that a displacement of chemical shift should not necessarily be anticipated for active site phosphorylated amino acid residues.

Only a sharp resonance was seen for succ-*P*, suggesting either a low affinity of the enzyme for this intermediate, or a high degree of flexibility of the phosphoryl group of enzyme-bound succ-*P*. Such flexibility might be expected for acyl phosphate intermediates, in view of the implied rotational freedom of the phosphoryl group of glutamyl-phosphate during catalysis by glutamine synthetase (39).

Our studies have provided insight to the role of the Mg^{2+} ion required for maximal enzyme activity. First the Mg^{2+} ion seems to be tightly complexed to the β-phosphoryl group of the enzyme bound AMPPCP (Fig. 7), perhaps a necessity for phosphoryl transfer from ATP to the histidine since phosphorylation by Mg-ATP occurs at a rate far exceeding that observed with ATP (13). Secondly, the Mg^{2+} ion seems to be close enough to the his-*P* residue to be involved in the phosphoryl transfer from histidine to succinate (Fig. 1). The Kd = 4 mM observed for this binding site suggests that this Mg^{2+} binding site is the one associated with that necesary for maximal catalytic activity (35). A second sphere metal ion complexation was suggested for the active site phosphoserine residue of phosphoglucomutase (38), and recent ^{31}P-NMR experiments of alkaline phosphatase have shown direct coordination of ^{113}Cd to Pi but not to the phosphoserine residue (40). All of these considerations support the idea of a direct role for the metal ions in phosphoryl transfer.

One way of summarizing our data is in the form of an energy diagram of the reaction as presented in Fig. 8. All three partial reactions are thought to have their own activation energy barrier. Formation of succinyl-CoA from

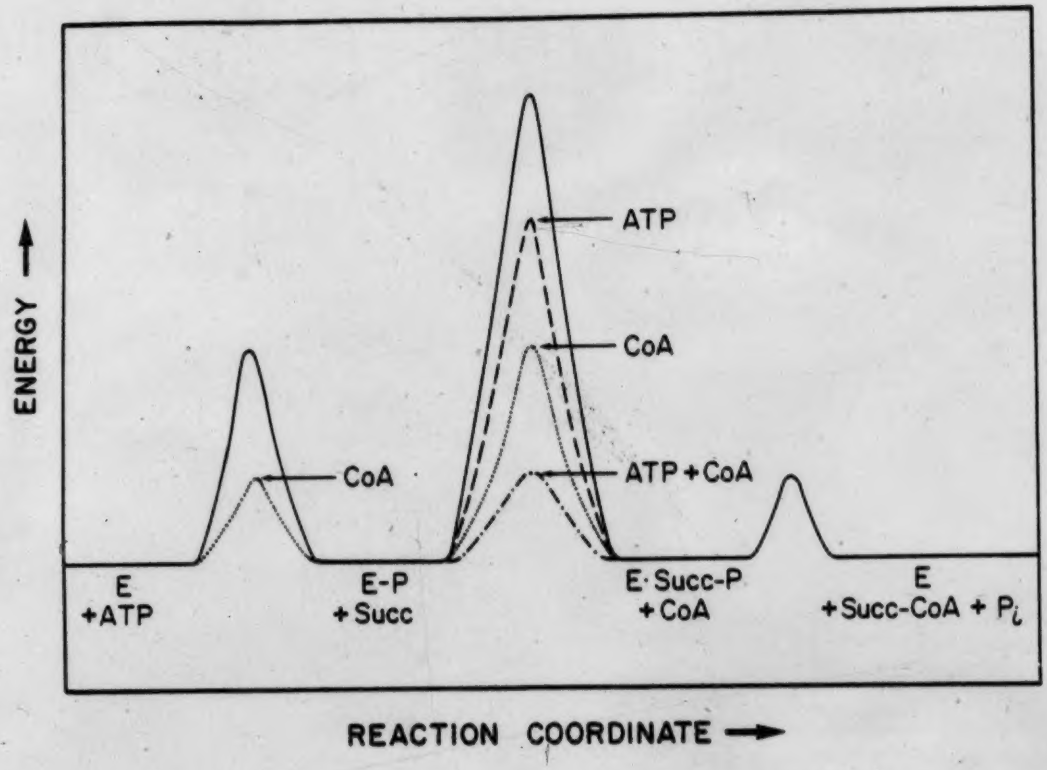
ENERGY
ATP
CoA
CoA
ATP + CoA
E
+ATP
E-P
+Succ
E·Succ-P
+CoA
E
+Succ-CoA + P_i
REACTION COORDINATE

Fig. 5.8 Schematic energy diagram for the succinyl-CoA synthetase reaction. For explanation see text. For the sake of clarity ΔGo is kept constant for the different states, although changes are known to occur (12).

succ-*P* appears to have a low activation energy since it occurs readily nonenzymatically (37). Partial reaction 2 does not occur by itself (Figs. 1,2,5) and therefore seems to have a very high energy barrier. We have shown that the phosphoryltransfer from histidine to succinate is strongly stimulated in the presence of CoA (Fig. 2) or ATP (Fig. 5). Analysis of the time course of these experiments indicated that CoA was more effective than ATP, hence it appears to cause a larger reduction in activation energy. It seems likely that the presence of both nucleotides will have an additive effect resulting in an even lower energy barrier. Partial reaction 1 is reported to be stimulated by Coenzyme-A nucleotides (6,7), possibly the change in the binding of AMPPCP observed after addition of CoA is related to this phenomenon (Fig. 7, Table 1). This can also be interpreted as a decrease in activation energy. Thus figure 8 shows that the overall reaction may proceed most readily when both nucleotides are present.

Addition of only succinate or its competitive inhibitor 2,2-difluorosuccinate has no effect on the chemical shift of the his-*P* resonance (Fig. 1). The presence of CoA causes a downfield shift in the his-*P* line that is additive with the Mg^{2+}-induced upfield shift. The CoA-induced broadening (Figs. 3B, 4D and 7C) can be most easily understood in terms of the scheme shown in Fig. 9. The his-*P* residue is on the smaller α subunit and the succinate and CoA binding sites

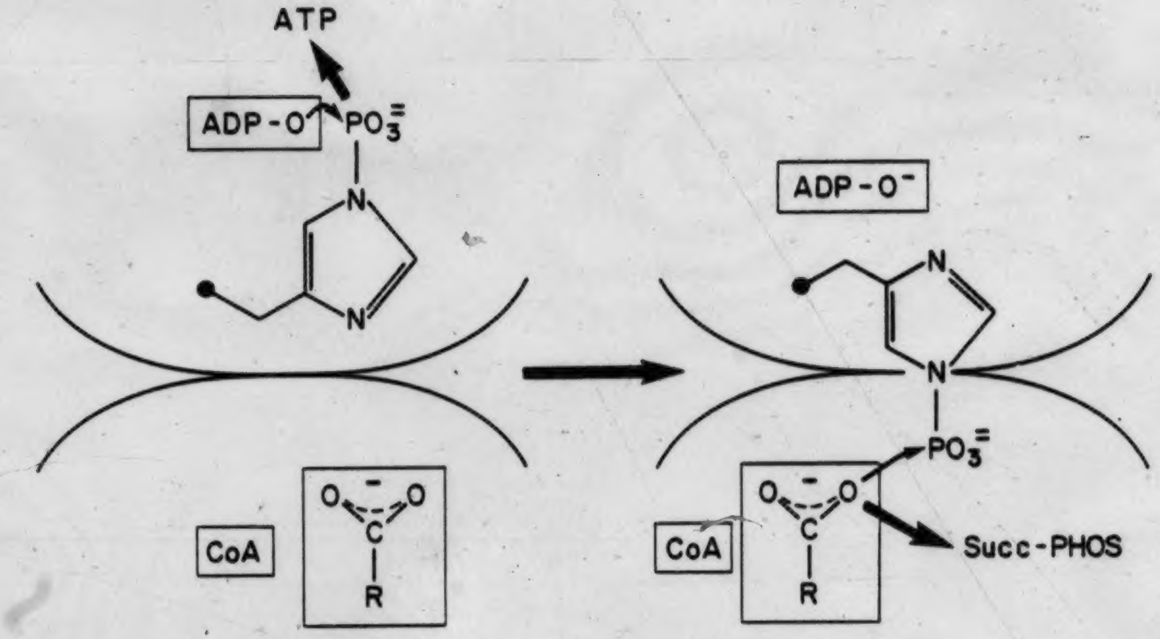
ATP
ADP-O
$PO_3^=$
N
N
CoA
O
O
C
R
ADP-O⁻
N
N
$PO_3^=$
O
O
C
R
CoA
Succ-PHOS

Fig. 5.9 Schematic representation of the active site of succinyl-CoA synthetase at the point of contact between the two subunit types. In this representation the his-*P* residue is visualized as being sufficiently mobile due to the binding of CoA to allow phosphoryl transfer either to ADP on the α subunit (left) or to succinate on the β subunit (right). See text for further explanation.

are on the β subunit (3-5). Binding of CoA is shown to permit at least two exchanging conformations for the his-*P*. One may allow for dephosphorylation by ADP and the other for transfer to the succinate residue to form succ-*P*. Such a mechanism is also consistent with CoA induced mobility as inferred from dansyl fluorescent label studies (J.S. Nishimura, personal communication). The presence of both succinate and CoA freezes the residue in one position ready for transfer (see Figs 3C and 7D). This agrees with the proton relaxation rate enhancement studies (28) that indicated a closed active site structure only in the presence of all substrates.

Our ^{31}P-NMR data showing that phosphorylation by ATP promotes succ-*P* production (see Figs. 5 and 6) can best be explained in terms of a catalytic cooperative model (41,42). Since both active sites are capable of catalysis (17), we favour an alternating site model over an asymmetric one-sided cooperative mechanism such as that recently observed for metal-deficient alkaline phosphatase (43). Our model is detailed in Fig. 10. The arrangement of the subunits is chosen according to crosslinking data (44), also the order of addition of substrates is consistent with the reported kinetic mechanism, which indicated binding of ATP first, followed by random addition of CoA and succinate (45). The dephosphorylated apoenzyme (left of figure 10) is clearly in a different conformation as indicated by its greatly

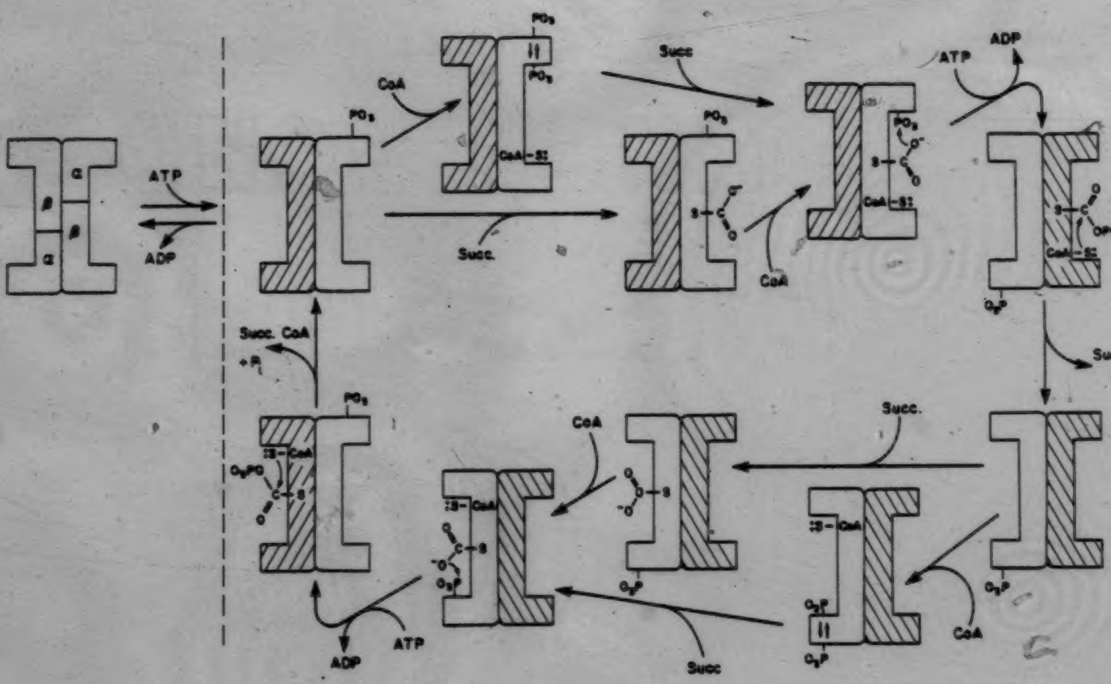
α
β
β
α
ATP
ADP
PO₃
CoA
Succ
Succ.
CoA-S:
ATP
ADP
OPO₃
Succ. CoA
+ Pi
O₃P
CoA
Succ.
:S-CoA
O₃PO
Succ
ATP
ADP

Fig. 5.10 Proposed model for alternating site catalytic cooperativity for *E. coli* succinyl-CoA synthetase. Different conformations for the two $\alpha\beta$ halves of the molecule are indicated by hatching and dots. Reciprocal conformational change is shown to be promoted by phosphorylation of one active site, accompanied by accelerated transfer of the phosphoryl group from the his-*P* to succinate at the other active site. See text for further explanation. Binding of ADP and Mg^{2+} has been omitted for the sake of clarity.

enhanced proteolytic susceptibility (13). Phosphorylation results in a different conformation characterised by two unequivalent active sites. The exchanging conformations induced by CoA and the subsequent locking by succinate are symbolized in the next set of diagrams (see also Figs. 3 and 8); the end result is not dependent on the order of the addition. This arrangement of substrates could by itself lead to slow product formation (Fig. 2C). However, since phosphorylation by ATP of one active site triggers a conformational change leading to phosphoryl transfer from histidine to succinate at the other, we propose the preferred route to be as indicated in Fig. 10. This requires phosphorylation of the heretofore non-active half and concomitant reciprocal change in conformation for both halves of the molecule. The last partial reaction then occurs quickly, leaving us with the reciprocal situation to where we started with the other subunit now phosphorylated. Clearly, going through the next catalytic cycle will regenerate the original enzyme form. This proposed scheme is consistent with all data presented here, together with initial rate kinetics (45,46), substrate synergism (1,7) and the Mn^{2+} binding studies (28,29). Although our scheme is drawn as if the enzyme is strictly half of the sites this is not an absolute requirement for this model. Negative cooperativity of the phosphorylation event would allow for the same interpretation (41,42). Our data clearly imply that the step in the reaction that is most sensitive to catalytic cooperativity is the transfer of phosphoryl from his-*P* to succinate. This is in contrast to the earlier proposals that ATP binding may facilitate release of succinyl-CoA (5,16). Furthermore, since phosphorylation of the enzyme is known to be exergonic ($G^{\circ}=-3$ kcal, Ref. 12), one can envision the proposed inversion of subunit conformation to be driven energetically by phosphorylation of the neighboring active site. This would not be possible for simple low-affinity binding of ATP to the phosphorylated enzyme (28,34).

Since CoA affects the binding of ATP analogues (Fig. 7, Table 1), and phosphorylation by ATP stimulates succ-*P* production on the opposite active site, attachment of CoA to both β subunits may be a prerequisite for optimal catalytic activity. Similarly the CoA-induced broadening effect (Figs. 3 and 4) becomes maximal only when both binding sites for this nucleotide are occupied. These considerations suggest that the originally observed CoA-induced enhancement of partial reactions termed "substrate synergism" is not necessarily mediated within one active site but could also be another manifestation of catalytic cooperativity between the two active sites. CoA-induced stimulation of GTP ↔ GDP exchange in the dimeric pig heart enzyme (47) is not easily explained in this way, however.

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CHAPTER 6

INHIBITION AND MODIFICATION STUDIES ON *E. coli* SUCCINYL-CoA SYNTHETASE

Summary

Several ATP analogues were tested as possible substrates in the *E. coli* succinyl-CoA synthetase reaction. The α,β and β,γ methylene-analog, the β,γ imido analogue as well as ATPγF could not substitute for ATP in the reaction. AMPPCP was a competitive inhibitor with ATP with a Ki = 0.38 mM. Malate as well as 2,2-difluorosuccinate were competitive inhibitors with succinate, with Ki = 4.5 mM and 0.9 mM respectively. Several ADP analogues were capable of inhibiting the SCS reaction assayed in the direction of succinyl-CoA formation but the natural nucleotide was most effective. A variety of tetrahedral anions could inhibit this same reaction. Arsenate was remarkably more effective than the natural product phosphate. The planar nitrate anions inhibited in a positive cooperative way. It is proposed that these can actually substitute for the covalent phosphohistidine intermediate. This observation is in support of earlier suggestions that one active site is sensitive to the phosphorylation state of the other. Studies using hybrid enzymes composed of active native subunits and inactive chemically modified subunits have indicated that species containing one modified β subunit per $(\alpha\beta)_2$ tetramer

are twice as active as the native species. Thus changes in subunit interaction can lead to an activation of catalysis, consistent with the idea of catalytic cooperativity.

Introduction

Most enzymes catalysing reactions pertinent to intermediary metabolism are composed of more than one subunit [1,2]. The majority of these are assembled of homologous subunit, but some enzymes are known to have a heterologous subunit structure [2]. For example *E. coli* succinyl-CoA synthetase has a tetrameric $\alpha_2\beta_2$ structure. The rationale for the presence of α as well as β subunits is well understood at present. Various studies have indicated that the binding sites for various substrates and products are on both subunits and that the active site is arranged at the interface of an α and a β subunit [2,3,4]. Thus the enzyme is best descried as a dimer of $\alpha\beta$ dimers. Therefore we will henceforth refer to the tetramer as $(\alpha\beta)_2$ rather than using the more common but less appropriate nomenclature $\alpha_2\beta_2$. It was recently demonstrated that no excess of either α or β subunits is found in *E. coli* suggesting that both subunits are synthesized in equal amounts [5]. However the rationale for the predominant existence of a tetrameric $(\alpha\beta)_2$ structure is far from clear at present. The enzyme purified from pig heart, for example, is reported to be composed of only one α and β subunit [6] and has a higher specific activity than the *E. coli* enzyme [7]. Recent experiments in our laboratory have indicated that at low protein concentration [8] and at high salt concentration the *E. coli* enzyme is capable of dissociating into $\alpha\beta$ dimers that resemble the pig heart enzyme with respect to specific activity and $P^{18}O$ exchange patterns [7]. Thus the $(\alpha\beta)_2$ tetramer could be viewed as an inhibited form of the more active $\alpha\beta$ dimer. It is further noteworthy in this context that both active sites do not act independently in the tetramer as was indicated by the half-of-the-sites phosphorylation observed for the enzyme [9,10]. These observations have triggered experiments which eventually led to proposals that the tetrameric enzyme has the property of alternating sites cooperativity [11] or less specific catalytic cooperativity [12]. Since both active sites are available for catalysis [13] the more esthetically pleasing alternating site models have been most emphasized [11,12]. However other possibilities have not been excluded up to date. For example, both active sites could react in a concerted fashion. Another possibility is that one active site could become phosphorylated, thereby stimulating its neighbour to become active while not being directly involved in catalysis itself. By analogy to the term "alternating sites" such models could be called "double-sites" or "single-site" catalytic cooperativity respectively.

Here we report on studies making use of hybrid tetramers of SCS containing chemically modified inactive subunits

[14]. A second approach we followed was inhibition studies with a variety of products and their analogues. Some results of such studies have been reported previously [15]. Moreover, we also examined a few substrate analogues to determine whether they could substitute for the natural compounds catalysing the reaction in the direction of succinyl-CoA-formation.

Experimental procedures

All chemicals used were obtained from commercial sources as described in Chapters 3 and 5. Sodium-orthovanadate was obtained from Alfa. In order to free ATPγF of contaminating ATP it was incubated with hexokinase, glucose and Mg^{2+} [16].

Succinyl-CoA synthetase was purified as described earlier [5]. It was judged pure by its behaviour as one band upon non-denaturing polyacrylamide gel electrophoresis. The enzyme was assayed at pH 7.2 in the direction of succinyl-CoA formation [9]. The assay mixture used contained 50 mM Tris-HCl, 10 mM $MgCl_2$ and saturating amounts of substrates. Inactive subunits were prepared by reaction of the native enzyme with NEM (0.2 mM) until no activity remained (1 hour). The modified $\alpha*$ and $\beta*$ subunits were subsequently purified by gel exclusion chromatography in acid urea as described for the native protein [5,17]. Hybrids were formed using the same conditions for refolding as normally used for the native protein [5,17]. These hybrids were checked for proper refolding by comigration with native protein on non-denaturing polyacrylamide gels.

Results

ATP analogues. A variety of ATP analogues were tested to determine whether they could substitute for ATP in the reaction at levels of enzyme 100-200 times higher than those normally used for assays. Since SCS catalyses cleavage of the β,γ phosphoryl linkage of ATP it was not surprising that both the β,γ imido- and methylene analogue did not act as a substrate. Less predictable was our observation that the $\alpha\beta$ substituted methylene analogue AMPCPP was also not a substrate for the enzyme. A wide variety of enzymes are capable of cleavage of the $\beta\gamma$ linkage of this analogue [16]. Moreover, AMPCPP closely resembles ATP with respect to Mg^{2+} complexation [18]. Thus the reason that it cannot act as a substrate is probably related to the difference in bond angles and bond lengths observed for pyrophosphate and its methylene analogue [16]. This illustrates the high precision of interactions between charged groups required to give rise to productive enzyme substrate complexes. We determined that the analogue AMPPCP β,γ was a competitive inhibitor with ATP with a Ki = 0.38 mM (Fig. 1). Moreover our ^{31}P-NMR studies have confirmed that it does bind to the enzyme [12]. For comparison, the substrate ATP has a Km = 0.02 mM, indicating that the binding site is better suited to bind the natural

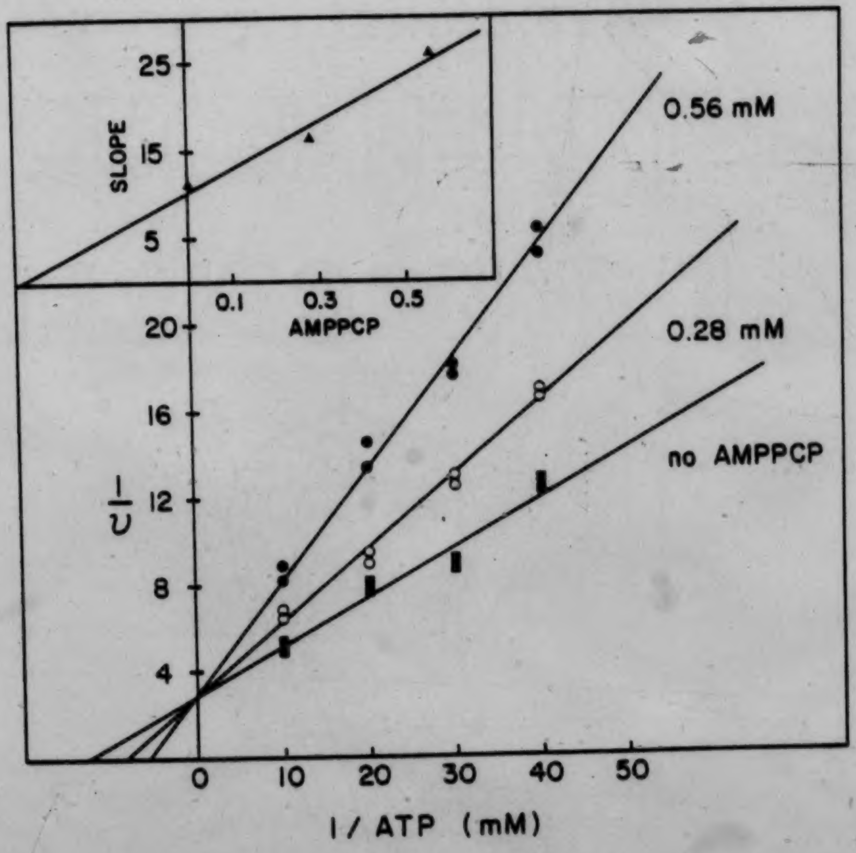

Fig. 6.1 Lineweaver-Burk plot of the AMPPCP inhibition of *E. coli* succinyl-CoA synthetase with ATP as the varied substrate.

compound than the analogue.

The fluorinated analogue ATPγF was also not a substrate for the enzyme, nor did its binding result in permanent inactivation of the enzyme as might have been inferred from the analogy to compounds like diisopropylfluorophosphate. The poor results generally obtained with this analogue [16] are probably a reflection of the fact that its metal binding properties differ strongly from those of ATP or other analogues [18].

Succinate analogues. A wide variety of organic acids have been tested to determine whether they could act as substrates [19] or if they inhibited the overall reaction in the direction of CoA formation [20]. Furthermore, a variety of such compounds was tested to establish their effectiveness in dephosphorylating the enzyme [21], and the acyl phosphates of most of these organic acids were synthesized to see whether they could support enzymatic production of ATP from ADP [22]. All these studies indicated that the succinate binding site is quite discriminatory. The only substitution that seems to be allowed is the attachment of an uncharged group at the α position. It is not fully clear whether such a substitution will end up in the α or β position with respect to the phosphoryl group, but the studies with the acyl-phosphate suggest that it could be in either position [20]. Significantly, fumarate exerted more effect than maleate [20,21], suggesting that a trans orientation of the two carboxyl groups is preferred. This also detracts from the appeal of the elegant anhydride type mechanism proposed by Spector and colleagues [23] for the succinyl-CoA formation from the succinyl-phosphate intermediate. A cis orientation of the carboxylates would be required for such an intermediate. Our studies have shown that the two α substituted succinate analogues malate (hydroxy-succinate) and 2,2-difluorosuccinate are competitive inhibitors with succinate, with Ki = 4.5 mM and Ki = 0.9 mM respectively. (The Km observed for succinate is 0.1 mM [3]). Malate is reported to give rise to some dephosphorylation [21]. Since 2,2-difluorosuccinate in our hands did not cause any dephosphorylation of the enzyme (as shown by ^{31}P-NMR) we have preferentially used this analogue over malate in our studies described in Chapter 5.

Inhibition by ADP and analogues. Results obtained by product inhibition studies with ADP and two of its analogues are shown in Figure 2. Assays were performed in the presence of saturating amounts of ATP, succinate and CoA. Under those conditions, quite low concentrations of ADP can have a drastic effect on the activity of the enzyme. Although the active site can accommodate both analogues (both inhibit), they are much less effective than the natural nucleotide. This again emphasizes the specificity of the enzyme's requirements for geometry and metal complexation for binding of nucleotides to the ADP/ATP site. Analysis of this ADP

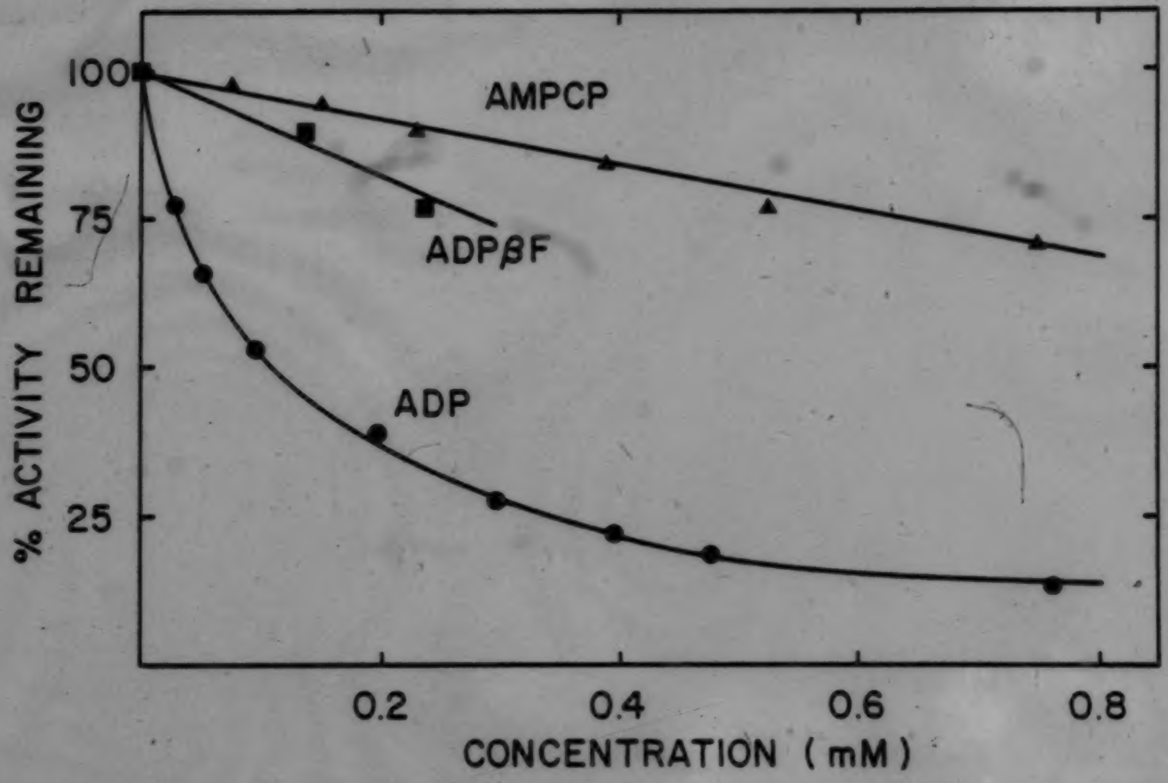

Fig. 6.2 Inhibition of *E. coli* succinyl-CoA synthetase by ADP and analogues. The activity in the absence of these inhibitions was arbitrarily set at 100%.

inhibition effect revealed straight lines in a reciprocal plot with an inhibition constant of 0.12 mM. The Km determined for ADP is 0.012 mM [3].

Inhibition by anions. We have compared the potency of a variety of anions in inhibiting SCS. As expected, the product inorganic phosphate inhibits the enzyme effectively (Fig. 3). Reciprocal plots of per cent of inhibition versus concentration of Pi gave rise to a straight line with an apparent Ki of 1.65 mM. The measured Km is 2.6 mM [3]. Significantly, other tetrahedral compounds [24] with four oxygen substituents also inhibited (Fig. 3,4). Both ClO_4^- and SO_4^{2-} were less effective than Pi. Arsenate was remarkably more effective than Pi (apparent Ki = 0.057 mM). Other anions such as acetate, formate and chloride were tested but had little effect on the enzyme's activity.[1]

Most remarkable is the cooperative inhibition pattern displayed by nitrate (Fig. 4). This behaviour clearly illustrates that both active sites do not act independently. In other studies the planar nitrate ions have been shown to be very effective in protecting creatine kinase from iodoacetamide inactivation, but only in the presence of creatine and ADP [24]. It was suggested this was accomplished by the formation of a transition-state analogue complex [24,25]

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[1] When we compared the effects of KCl, NH_4Cl and LiCl, we found that at a concentration of 175 mM these compounds inhibited by 15%, 25% and 35% respectively. Thus for our studies with other anions we preferentially used the potassium salts.

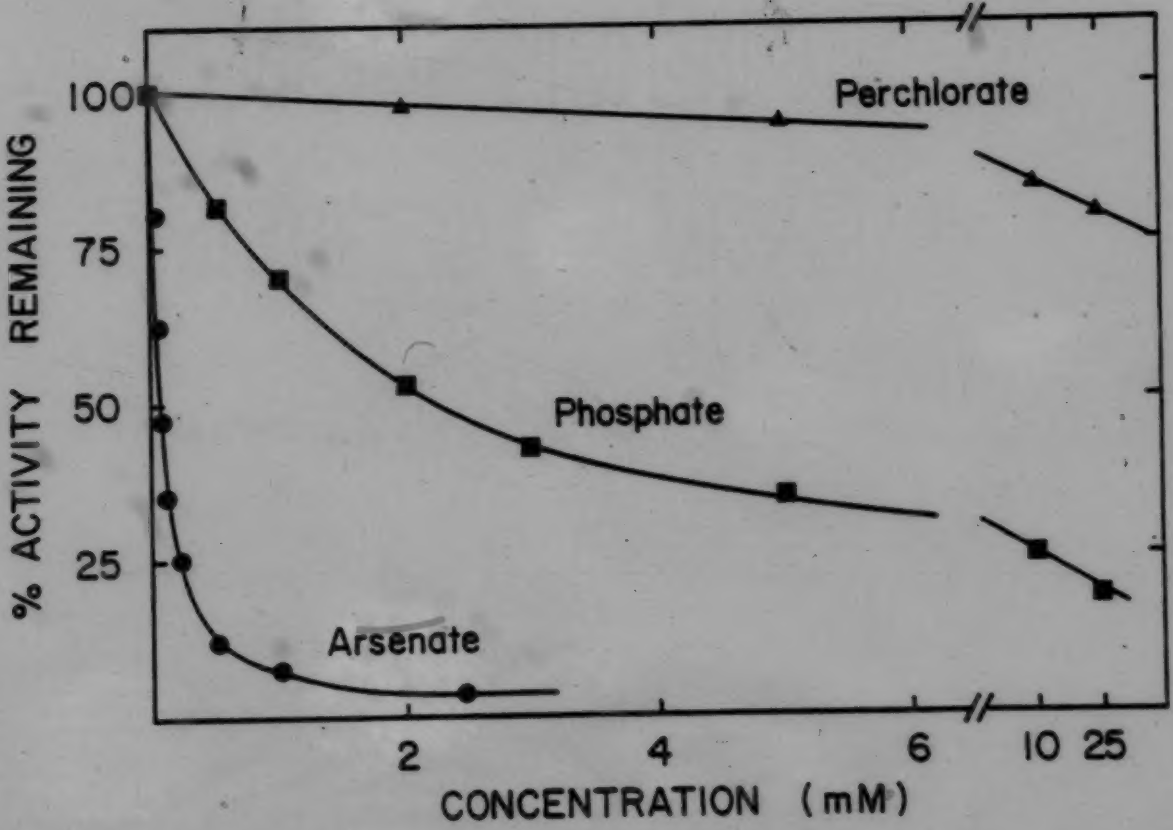

Fig. 6.3 Inhibition of *E. coli* succinyl-CoA synthetase by anions. The activity in the absence of the anions was arbitrarily set at 100%.

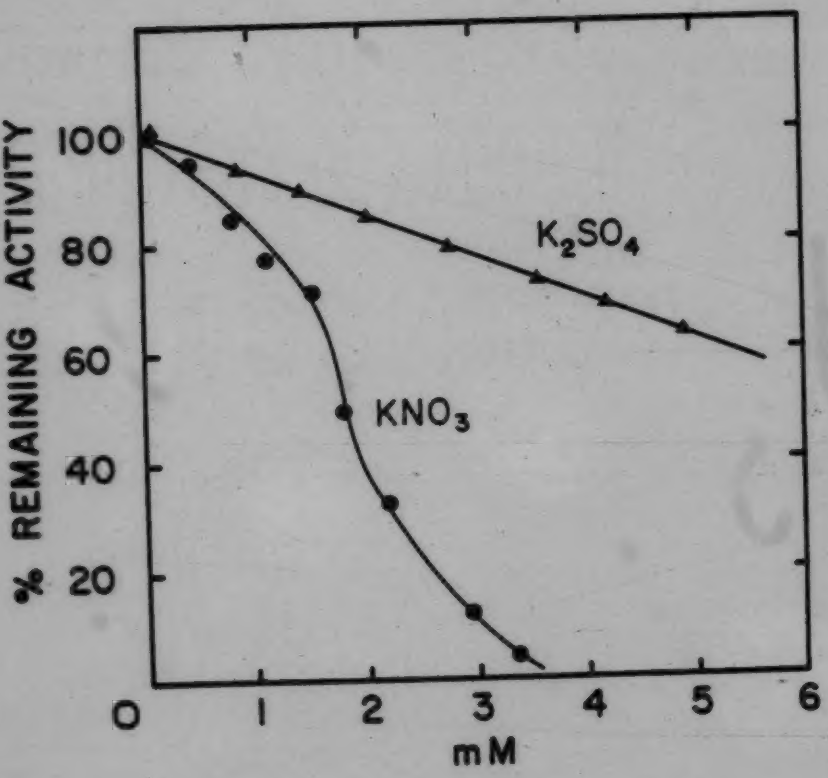

Fig. 6.4 Inhibition of *E. coli* succinyl-CoA synthetase by anions. The activity in the absence of the anions was arbitrarily set at 100%.

where the planar nitrate ion mimics either a planar trigonal metaphosphate intermediate as in dissociative mechanisms or part of a trigonal bipyrimidal intermediate as in associative mechanisms [24]. When both ADP and NO_3^- were added together to SCS their inhibitions were only additive and did not result in the drastic decrease in activity that is expected for a transition state analogue complex. Experiments with nitrite (which is planar like nitrate [25]) and vanadate (which has a trigonal bipyrimidal structure and has been shown to inhibit several phosphoryl transferring enzymes [25,26]) were unsuccessful since both interfered with the assay.

Studies using hybrids. As outlined by Meighen and Schachman [14] three different types of hybrids can be constructed. One class is reconstituted from a mixture of naturally occurring subunits purified from different organisms. Other approaches make use of either mutationally altered or chemically modified subunits. Since the genetics of *E. coli* SCS have not yet been investigated, mutationally altered subunits are not presently available. It was recently shown that SCS purified from *E. coli* and pig heart were antigenetically unrelated [28]. This observation decreases the likelihood that hybrids containing subunits of each of these enzymes could be formed. Recent experiments in this laboratory have shown that such hybrids are indeed not formed (W.T. Wolodko, unpublished observations). Here we will report on studies with hybrids composed of active native subunits and inactive NEM modified subunits. These studies were performed several years ago by Mr. E. Brownie and are presented here since they can be understood in terms of the model presented in Chapter 5.

Fig. 5 shows the inactivation reaction of native *E. coli* SCS by 0.2 mM NEM. The enzyme is fully inactivated in one hour. Since the modification is performed on intact enzyme rather than on the separated subunits it may be expected that such preparations still have intact subunit contact regions and that inactivation arises from modifications elsewhere, such as at the active site.[1] Subunits prepared from enzyme treated in this fashion were used for refolding experiments with the following outcome. Refolding of native α and β subunits usually results in almost complete recovery of activity and phosphorylation capacity [5,17]. Enzyme containing only $\alpha*$ and $\beta*$ subunits were inactive and could also not be phosphorylated. Hybrids made up of equal amounts of $\alpha*$ and β or α and $\beta*$ were both inactive but could be phosphorylated. Thus these results confirm that the presence of both subunits is a necessity for overall catalysis to occur [17]. Most intriguing however was the outcome of the experiment depicted in Fig. 6. Here

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[1] Some mercurials give rise to a dissociation of the enzyme [9,29]. We have investigated the enzyme for this property by sedimentation velocity experiments. These showed that NEM-modified and native enzyme behaved as expected for the $(\alpha\beta)_2$ tetramers supporting the idea that the subunit contact domains are still intact.

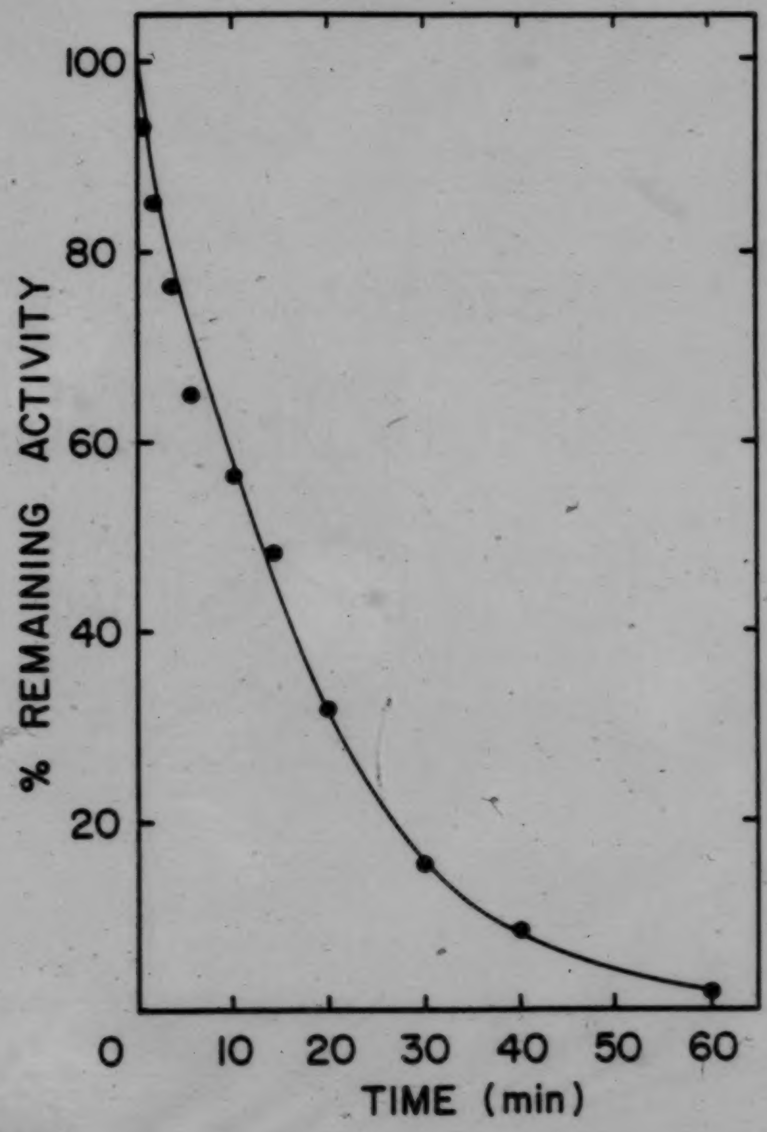

Fig. 6.5 Inactivation of *E. coli* succinyl-CoA synthetase by NEM. A solution of approximately 0.5 mg/ml of enzyme (Tris HCl 40 mM, pH 7.2, 0.1 M of EDTA was incubated with 0.2 mM NEM. Small aliquots were withdrawn at certain time intervals and were added to 3 ml of assay medium containing 1.0 mM B-mercaptoethanol. These mixtures were assayed for activity. The graph shows the decrease in the log of the activity in arbitrary units with time.

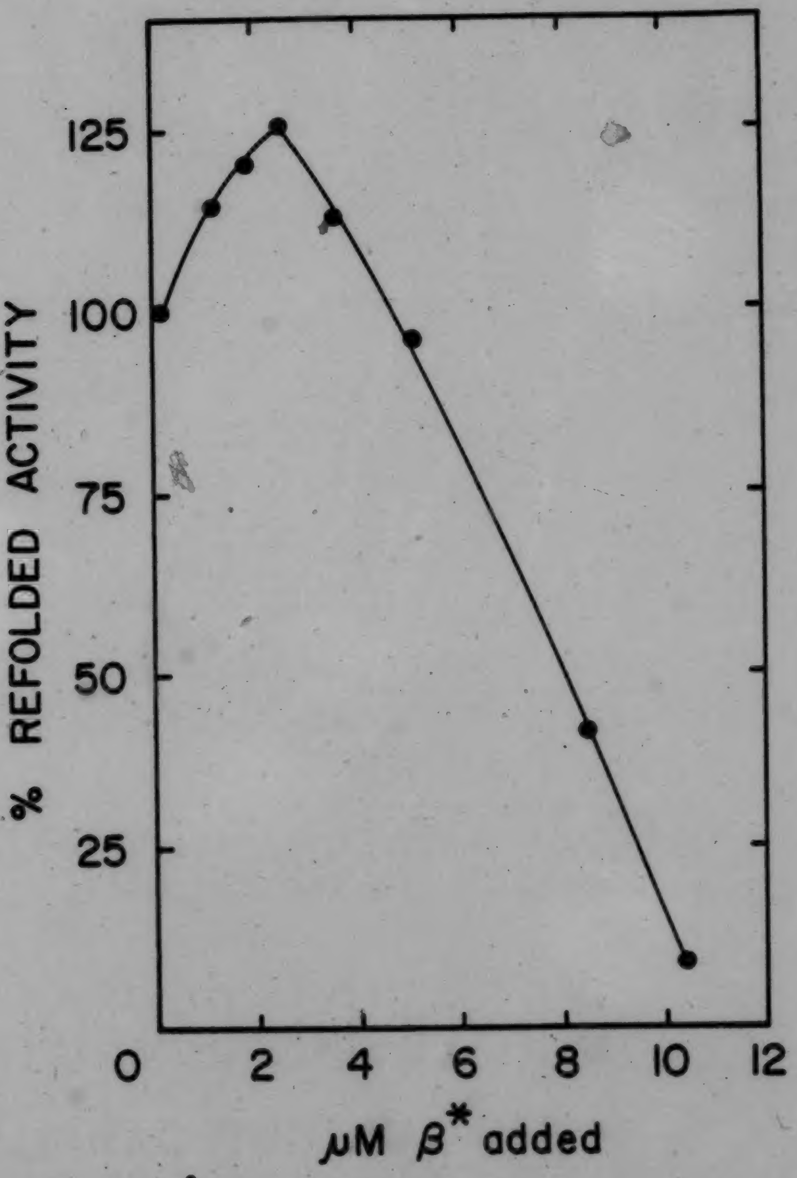

Fig. 6.6 Activity of hybrids of *E. coli* succinyl-CoA synthetase refolded with different amounts of inactive modified β subunits (β*). The activity measured in the absence of any added β* was arbitrarily set at 100%. The incubation mixture contained 1.5 umol of α and β subunits in addition to the β* that was added.

we show that addition of $\beta*$ to 1:1 mixture of α and β subunits can actually lead to an activation of the enzyme. Thus although $(\alpha*\beta*)_2$ is fully inactive the species $\alpha\beta\alpha\beta*$ shows a higher activity than observed for $(\alpha\beta)_2$. It appears that $\beta*$ is frozen into a conformation that confers a higher activity onto the next active site. If we make the assumption that we have obtained a random distribution of hybrid-species, at most only half of the molecules will be $\alpha\beta\alpha\beta*$, in which case these must be at least twice as active than the $(\alpha\beta)_2$. It appears that β refolds more efficiently than $\beta*$, providing an explanation for the fact that the peak of activity is observed at a ratio of approximately $\alpha:\beta:\beta* = 1:1:2$. The subsequent decrease in activity when more $\beta*$ is added must be attributed to the predominant formation of the inactive $(\alpha\beta*)_2$ species.

Discussion

Our studies have shown that the majority of the analogues tested bound with less affinity than the natural compounds. The only exception was arsenate which was far more effective as an inhibitor of the enzyme than phosphate. This in turn was more effective than sulfate or perchlorate. It appears that arsenate fits most snugly into the phosphate binding pocket and the differential inhibition seen with these anions is presumably a reflection of a difference in size since they all resemble a tetrahedral structure [25]. Most remarkable however was the cooperative inhibition pattern observed with nitrate ions. Since addition of low concentrations of both ADP and NO_3^- did not result in a dramatic decrease in the activity of the enzyme, we think it is unlikely that this combination simulates the phosphoryl group transfer from ATP to histidine. Rather we feel that the nitrate anion mimics the phosphoryl group of the phosphohistidine in one of its conformations [12]. In support of this is the fact that the enzyme bound phosphohistidine residue appears to be in the monoanion form [30]. Also the planar formate ion could not substitute in the inhibition of SCS for the nitrate ion. Thus a planar orientation of three oxygen atoms with only one of the three negatively charged seems to be a requirement for the effect. Such a structure resembles the bottom of the tetrahedral structures of a monoanionic phosphohistidine residue. Moreover we have provided evidence showing that phosphorylation of one active site may trigger catalytic events at the other [12]. Thus the positive cooperative nature of the nitrate inhibition can be interpreted as another manifestation of one active site being aware of the phosphorylation state of the other. These results are also consistent with our earlier notion that both halves of the enzyme molecule are made unsymmetrical by phosphorylation [12]. It has also been shown that ADP inhibition at subsaturating ATP levels leads to non-linear Lineweaver-Burk plots [15]. All these observations, taken together with the half-of-the-sites phosphorylation, [9,10] clearly indicate that the two active sites do not act fully independently and are capable of communicating with each other.

Further support is provided by our experiments with the hybrid enzyme. Since the species $(\alpha\beta*)_2$ was inactive it seems reasonable to assume that the $\alpha\beta*$ half of an $\alpha\beta\alpha\beta*$ species will be inactive.[3] Although it is inactive it still contributes to catalysis by activating the $\alpha\beta$ half of the molecule to twice its usual activity. Possibly the $\alpha\beta*$ half of the tetrameric enzyme molecule is frozen by the modification into a conformation that resembles the phosphorylated half of the enzyme (see Fig. 10 of Chapter 6) and is thus capable of activating its neighbour. One might argue that since half of the active sites of the $\alpha\beta\alpha\beta*$ molecule is thought to be inactive in this hybrid that this result is incompatible with "alternating sites catalytic cooperativity" but supports a "single-site model". This suggestion is very premature, however, and awaits a careful comparison of specific activities under a variety of experimental conditions. Thus the minimal conclusion from this experiment is that changes in the subunit interface result in changes in activity in the active site composed of native subunits. This is in support of the general concept of catalytic cooperativity [12].

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[3] This assumption deserves further experimental confirmation. For example, the integrity of the binding sites for CoA and ADP in this species could be investigated.

These results also gave some more insight into the rationale for the existence of an $(\alpha\beta)_2$ structure. The results with the hybrids especially show that our earlier suggestion that $(\alpha\beta)_2$ is an inhibited form of the $\alpha\beta$ dimer is correct. Apparently certain conformational changes can promote a change from a rigid tetramer (T) to a "loose tetramer" (R) (similar to the $\alpha\beta\alpha\beta*$ hybrid) which resembles the pig heart enzyme, as well as the dimer observed for the *E. coli* enzyme in high salt concentration [7]. At first sight it may seem unusual that an organism would want to make an enzyme less effective. However, the fact that allosteric regulation may have a similar effect on the activity of other enzymes is generally accepted. Since the activity of this enzyme is not open to allosteric regulation, evolution may have developed an alternative means of regulation. If the level of products or substrates (for example Pi may promote formation of the tetramer [7]) could result in a shift between a highly active "dimer-like" loose tetramer structure (R) and a rigid tetramer with low activity (T), this would provide an attractive means for non-allosteric regulation of the activity of the enzyme which would be more effective than product inhibition. Moreover, catalysis is not blocked in the rigid tetramer, but is only less effective than in the "dimer-like" enzyme. Catalysis in this species proceeds in a catalytic cooperative way as indicated in the previous chapter. The conformational changes required for this are likely to be similar to those for the T to R transition. It would be of interest to determine if both proposed tetrameric structures catalysed the forward and reverse reactions with equal efficiency. Another rationale for the complexity of the subunit structure of *E. coli* SCS could possibly be that different states may favour a different direction of the overall reaction.

In Table 6.1 we present a list of the properties that the proposed T and the R conformation appear to have. This list explains some of the discrepancies that exist between results published by different laboratories or sometimes within one laboratory. For example, we have shown [12] that the presence of succinate and Mg^{2+} does not dephosphorylate the enzyme, whereas others in our laboratory have shown the opposite [10]. The only obvious difference between both experiments is a difference in protein concentration. Such discrepancies can be explained by the arrangement of properties for the T and R state tetramers as shown in Table 1. Since the mitochondrial pig heart enzyme is purified as a dimer [6] it appears as if this enzyme would escape a similar control. However effects of "macromolecular crowding" are reported to have drastic effects on the activity of multisubunit enzymes [31,32]. Such effects are thought to be mediated by shifts in monomer, dimer, tetramer associations. Especially since at high protein concentrations the pig heart enzyme resembles the *E. coli* enzyme with respect to $P^{18}O$ exchange [7], we feel that a similar regulation may play a role in mitochondria.

Table 6.1

Properties of the two forms of E. coli succinyl-CoA synthase

"dimer like" tetramer (R)	rigid tetramer (T)	Ref.
low protein concentration	high protein concentration	8
high activity	low activity	7
stabilised by high salt conc.	stable at low salt conc.	7
low $P^{18}O$ exchange	high $P^{18}O$ exchange	7
resembled by NEM modified species	--	-
--	studied by ^{31}P-NMR	12
both sites phosphorylated	half-of-the-sites phosphorylated	12
can be dephosphorylated by succinate and Mg^{2+}	cannot be dephosphorylated by succinate and Mg^{2+}	10,12
stabilised by nitrate?	stabilised by phosphate	10
labile to proteolysis?	stable to proteolysis	10

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CHAPTER 7

^{1}H NMR STUDIES OF *E. coli* SUCCINYL-CoA SYNTHETASE.

Summary

The ^{1}H NMR spectrum of the tetrameric $(\alpha_2\beta_2)$ *E. coli* succinyl-CoA synthetase (MW=140,000) was obtained at 270 MHz. Although broadening due to slow overall tumbling of the protein molecule caused superposition of most of the residues, one well resolved C2 histidine resonance, probably representing 2 similar histidine residues, could be observed. The pH-dependence of its chemical shift indicated an apparent pKa of 6.70. Since addition of the substrate CoA, or of the metal cofactors Mg^{2+} or Mn^{2+}, or dephosphorylation did not cause any changes in this resonance, we concluded that it represents neither the catalytic phosphohistidine residue nor the imidazole group implicated in the generation of the CoA nucleophile. Instead, it probably represents a surface residue that is signalled by the linewidth to have considerable freedom of motion. This mobility seems enhanced in the absence of phosphate ions, which is also reflected by a decrease in linewidths seen for other unresolved aromatic residues and is consistent with reports showing that phosphate ions stabilise the tetrameric $\alpha_2\beta_2$ form over the dimeric $\alpha\beta$ form and protect the enzyme from proteolytic degradation. The presence of 6 M urea completely unfolded the protein.

Introduction

E. coli succinyl-CoA synthetase is an $\alpha_2\beta_2$ tetrameric enzyme with two active sites each arranged at the interface of an α and β subunit and an overall molecular weight of 140,000 [1,2]. Each enzyme molecule contains a total of 18 histidine residues with 5 located in each α subunit and 4 in each β subunit [1]. Two out of every 9 imidazole groups per $\alpha\beta$ dimer are called upon for a catalytic role. The first one, located in the smaller α subunit [3,4], serves as a nucleophile and generates the covalent phosphohistidine intermediate from ATP [1,2]. The second catalytic histidine is part of the β subunit and has been proposed to act as a general base stabilizing the cysteinyl-S^- nucleophile responsible for driving the succinyl-CoA and Pi formation from the noncovalent succinyl-phosphate intermediate [5].

Here we will report on a 270-MHz ^1H NMR study aimed at resolving the histidine residues of succinyl-CoA synthetase. Proteins with molecular weights in excess of 50,000 are expected to exhibit slow overall tumbling so that resolution of individual resonances in a ^1H NMR spectrum should be impossible. However, it was recently demonstrated that reasonable spectra could be obtained for large proteins such as human IgG immunoglobulins (MW = 152,000) [6], pyruvate kinase (MW = 237,000) [7], myosin (MW = 450,000) [8] and even for viruses and ribosomes [9]. Such measurements indicate internal mobility in certain parts of the protein molecule. For example, in the case of the immunoglobulins the His-224 resonance located in the flexible hinge region could be assigned [6]. For pyruvate kinase the spectra even allowed the resolution of 6 of a total 14 histidine residues; one of these was perturbed by substrate additions and could therefore be assigned a role in the active site of this tetrameric enzyme [7].

Experimental procedures

Most procedures used were as outlined earlier for our ^{31}P-NMR studies of the same enzyme [10], with a few small changes. Samples (2.5 - 8 mg/ml final concentration) were precipitated with $(NH_4)_2SO_4$ (enzyme grade) and then dissolved in a buffer (pH 7.0, 50 mM KH_2PO_4, 0.05 mM EDTA or 0.1 mM EDTA, 99.8% D_2O (Biorad). Afterwards they were dialysed overnight against the desired buffers all made up in 99.8% D_2O. The pH was measured with a radiometer pH meter equipped with a glass electrode. No corrections were made for the D_2O in the buffer.

^{1}H NMR spectra were obtained at a frequency of 270 MHz on a Bruker HXS-270 spectrometer operating in the Fourier transform mode. Pulses of 20 usecs (80°) were used; the time between individual pulses was 1 sec. Individual FID's were stored and added in an 8K size computer memory, using block averaging. Spectra were obtained with homonuclear broadband decoupling to suppress the HDO-resonance. Normally 25,000 acquisitions for a 2.5 mg/ml sample, or 8,000 scans for a 8 mg/ml sample were obtained. Samples of 1.5 ml were placed in 10 mm precision tubes (Wilmad), equipped with Teflon-Vortex plugs. The sample temperature was controlled at 27°C. Chemical shifts are given relative to an internal standard of DSS (4,4 dimethyl-4-silapentane-1-sulfonic acid) and downfield shifts are given a positive sign.

Results

^{1}H NMR spectrum. Figure 1 shows a representative spectrum obtained for a 2.7 mg/ml solution of phosphorylated succinyl-CoA synthetase. This spectrum demonstrates that certain segments of the protein have internal mobility but that the protein has a defined folded tertiary structure. This last fact becomes obvious from a comparison of the spectrum in figure 1 with that obtained for a sample that was denatured in 6 M urea (pH 7.3) (data not shown). Under those last conditions the tyrosine 3,5 and 2,6-protons, for example, appear as well resolved narrow lines at 6.9 and 7.2 ppm respectively, whereas in the folded protein only a shoulder on the aromatic region peak at 6.8 ppm indicates their presence. Moreover the resonances observed in the aliphatic region of the spectrum (0-4.7 ppm) and those seen for the phenylalanine and tryptophan protons appear in the 6 M urea spectra as a collection of narrow lines at positions characteristic of amino acids freely tumbling in solution, indicating that no tertiary structure is left

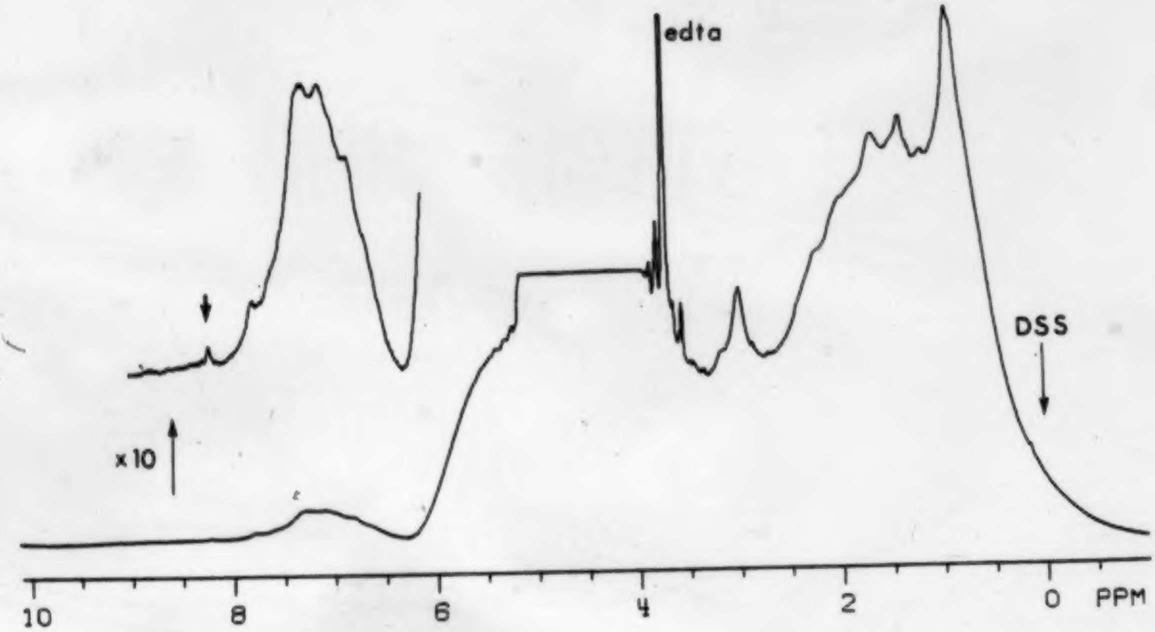

Fig. 7.1 270 MHz 'H NMR spectrum of 2.7 mg/ml *E. coli* Succinyl-CoA synthetase (Exponential multiplication 1 Hz). Conditions were as described under experimental procedures.

under these conditions. In figure 1 for the native protein the broad peaks centered around 7.1 and 7.3 ppm originate from the tyrosine 2,6, tryptophan, phenylalanine and histidine C4 proton resonances. The shoulder appearing at 7.8 ppm most likely arises from histidine C2 protons and the sharp resonance at 8.25 ppm (see arrow) originates also from an imidazole C2 proton.

The enzyme contains a total of 370 aromatic protons [1]. Comparison of the intensity of the small peak at 8.25 ppm to the other aromatics showed an intensity of about 1.5 - 2.5 residues for three different spectra taken at pH 7.0. This indicates that only two histidine residues contribute to the signal, although it should be pointed out here that such measurements are complicated by the presence of the broad HDO resonance which was not fully suppressed, cross-relaxation effects due to the decoupling, overlap with unexchanged amide-hydrogen resonances and errors in the set up of the spectrometer (i.e. pulsing faster than is necessary to allow for full recovery of all nuclei).

pH dependence. In another set of experiments, carried out on samples of 7 mg/ml of enzyme, we determined the pH-dependence of the observed signals in the aromatic region of the spectrum. Unfortunately, the enzyme has a narrow stability and activity profile centered around pH 7.2. Therefore we could only vary the pH between 6.5 and 8.0. In this range some small changes occurred in the area of the aromatic peak representing the phenylalanine and tryptophan residues, possibly indicating pH dependent structural rearrangements (data not shown). The resonance at 7.8 ppm, assigned to histidine C2 protons, however did not significantly shift, suggesting that most of the histidines do not seem to be deprotonated. The only resonance that shifted was the one originally centered at 8.25 ppm (pH [illegible].0). Normally the chemical shifts of a histidine C2 proton shifts over about 0.9 ppm in a pH titration experiment [14]. Figure 2 clearly shows that a "normal" titration curve for this residue can be drawn suggesting a pKa of 6.70.

Addition of substrates. Since ^{31}P-NMR studies had clearly shown an upfield shift for the phosphohistidyl resonance upon Mg^{2+} addition and that addition of CoA resulted in extensive broadening of that resonance [10] we checked the influence that additions of either one of these compounds had on the resolved histidine residue. The enzyme concentration for these experiments was 8 mg/ml. Additions up to 1 mM (2 mM DTT was included with CoA) did not result in any observable changes in the aromatic part of the spectrum. Moreover the presence of both Mg^{2+} and succinate, known to cause dephosphorylation of the enzyme [11], did not result in obvious differences in the proton NMR spectra. Addition of 50 uM paramagnetic Mn^{2+}, which can substitute for Mg^{2+} in the active site [1,10] caused broadening of the resolved C2-histidine resonance. However, all other non-

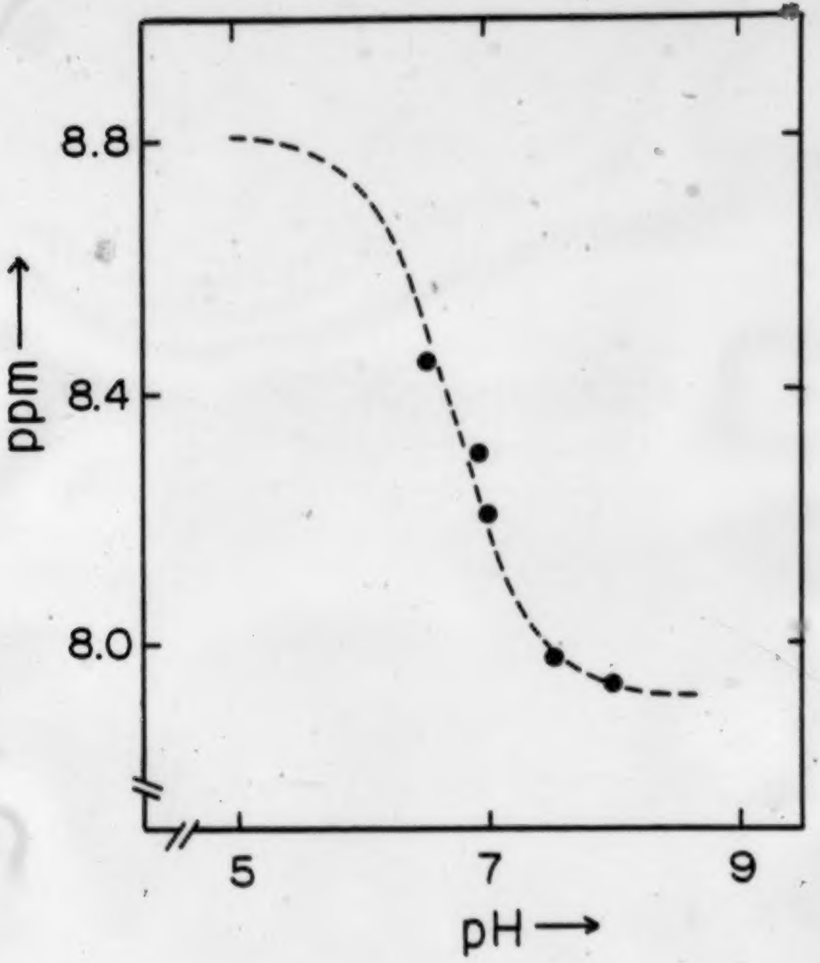

Fig. 7.2 pH-dependence of the resolved C2-histidine resonance. Samples with concentration of 7.5 mg/ml were used. Other conditions were as described under experimental procedures.

resolved aromatic resonances were broadened to a similar extent, indicating that this was a non-specific effect and thus not caused by specific binding of the metal ion in the active site. In the course of these experiments we noticed that omission of the phosphate ions resulted in a sharpening of the resolved histidine C2-peak and of most of the aromatic resonances observed. Such an effect signals increased mobility.

Discussion

Since at neutral pH histidine can serve as a potent nucleophile and base it is often found in the active site of enzymes. If such a residue can be resolved in a ^{1}H-NMR spectrum, valuable mechanistic information can be obtained as exemplified for a variety of other phosphoryl transferring enzymes: pyruvate, adenylate, creatine kinase [7,12,13] and ribonuclease [14]. Unfortunately, our results obtained here indicate that the only resolved histidine C2 resonance observed is a surface residue and does not serve a catalytic role. Our pH titration studies could not establish this since titration curves obtained for histidine and N-3 phosphohistidine standards were virtually identical [15]. But while addition of substrates and metal ions exhibited no specific effects here, ^{31}P-NMR measurements indicated significant changes for the phosphohistidine residue (10). ^{31}P-NMR measurements have also indicated that the phosphohistidine residue is rigidly held [16], rendering it unlikely that the observed narrow histidine C2-resonance represents the same residue. Moreover, the observed stoichiometry (2 identical histidines per molecule) makes it unlikely that we deal with the phosphohistidine residue since only one α subunit is phosphorylated at any time [1,2,11]. For similar reasons the observed histidine C2 resonance does not appear to originate from the imidazole-group stabilizing the cysteinyl nucleophile [5]. The pKa of 6.70 is in the same order as those usually observed [14] and can be taken as an indication that the titratable hydrogen is probably not involved in a salt linkage.

Chemical modification studies with ethoxy formic anhydride have shown that only 2-3 histidine residues out of a possible 9 per αβ dimer are exposed [5]. This seems consistent with our observation that the majority of the histidines is shielded since they do not exhibit an expected change [14] in their chemical shift (7.8 ppm) between pH 6.5 and pH 8.0 and therefore seem shielded from the solvent. Usually histidine-protons have long T_2's resulting in narrower resonances than those observed for most other aromatic protons [17]. This makes it unlikely that they are too broad to be observable and would thus have escaped our attention.

The increase in mobility seen in the absence of phosphate ions could be the result of the decrease in ionic strength. The presence of chloride ions, however, has only small effects on the activity and stability of the enzyme whereas phosphate ions markedly protect against proteolytic degradation [11]. Sedimentation equilibrium studies have indicated that low concentrations of phosphate ions favour the $\alpha_2\beta_2$ tetramer form of the enzyme over the $\alpha\beta$ dimer [18]. Thus the changes we observed could be related to these phenomena. Since succinyl-CoA synthetase exhibits the properties of substrate synergism and catalytic cooperativity [1,2,10] large scale molecular motions are necessary to provide for such intra- and intersubunit communications. The internal mobility observed here may be a reflection of such conformational flexibility similar to that observed for other proteins [6,7,8,9].

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CHAPTER 8

^{31}P-NMR pH TITRATION STUDIES OF THE PHOSPHOPROTEINS TROPOMYOSIN AND GLYCOGEN PHOSPHORYLASE *a*

Summary

^{31}P-NMR pH titration studies of the phosphoproteins tropomyosin and glycogen phosphorylase *a* (in the presence of the inhibitor glucose) show that the resonances observed for the serine phosphate regulatory sites shift with pH. Analysis of linewidths indicates that both residues have some mobility. These results are in agreement with studies on similar phosphorylated sites on other proteins, suggesting that mobility may be a general property of such regulatory sites. pH titration studies on a series of model compounds have indicated that an empirical correlation exists between the Hill coefficient (a measure of the cooperativity of the titration curve) and the presence of charged groups in the vicinity of the phosphoryl moiety. Moreover these studies showed that within one class of similarly substituted phosphorus compounds, the chemical shifts, the titration behaviour and the pKa_2 were comparable and allow for easy identification of these compounds. The pKa_2 observed for the protein bound serine-phosphate residues are in general a bit higher than those of phosphomonoester-containing small compounds.

Introduction

Protein phosphorylation plays an important role in the regulation of enzyme activity in intermediary metabolism [1] and is also thought to play a role in the regulation of muscle contraction [2], in the control of DNA transcription and replication via histone phosphorylation [3] and in the process of certain viral transformations [4]. Such reversible covalent modifications are performed by protein kinases and phosphatases and occur on specific serine residues [1]. Other phosphoamino acids have also been implicated for such functions, including the phosphomonoesters phosphothreonine and tyrosine [4], the phosphoramidates phospholysine [5] and N-3 and N-1 phosphohistidine [5,6], the acylphosphate, aspartylphosphate and phosphodiesters like 5'-adenylyl-O-tyrosine [7]. To understand the mechanisms by which such structural phosphorylations can exert their regulatory functions, knowledge of the mobility and protonation states of the phosphate moiety will be helpful.

^{31}P-NMR can provide this information. Unlike normal pH titration, it is not hampered by ambiguities about the assignment of observed pKa's to the phosphoryl group and also the instability of certain bonds at pH extremes can be readily detected and does not interfere with a determination of the pKa. Although in several cases the protonation state of the phosphoryl moiety has been directly deduced from the chemical shift it has been pointed out that such practice should be discouraged since factors other than the protonation state may cause changes in the chemical shift [8]. In another report [9] we have shown that information about the mobility and protonation state can be obtained in special cases from a study of the frequency dependence of the linewidths.

Here we will report on ^{31}P-NMR pH titration studies performed on a series of model compounds. A variety of such studies have been reported [10-14] but complete titration curves were shown for only a few cases. Moreover we performed detailed pH titration studies on the phosphoproteins tropomyosin and phosphorylase *a*, and some results obtained with phosvitin and ovalbumin will be reported as well. These studies have allowed us to determine the effects of charged groups in the vicinity of the phosphoryl moiety on its titration. Our observations are in agreement with similar studies reported for other phosphoproteins [14,15] and have revealed an interesting common feature of all regulatory sites.

Experimental procedures

Tropomyosin ($\alpha\alpha$) was prepared from rabbit skeletal muscle and was a gift from Ms. E.M. Lohmeier and Dr. L.B. Smillie. Samples (60 mg/ml) were dissolved in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 10 mM DTT, 25% D_2O. Phosphorylase *a* was prepared from rabbit skeletal muscle and was a gift from Mrs. S. Shechosky and Dr. N.B. Madsen. Samples (25 mg/ml) were dissolved in a buffer containing 50 mM Tris-HCl, 50 mM MES, 100 mM glucose, 1 mM EDTA, 2.5 mM DTT and 50% D_2O. All model compounds studied were obtained from Sigma Chemical Company. Phospho-L-arginine, phosphocreatine, phospho-enol-pyruvate and carbamyl-phosphate were obtained as their respective sodium salts. DL-O-phosphothreonine, O-phospho-L-serine and adenosine 5'-monophosphoramidate were purchased in the acid form and acetyl-phosphate as the lithium potassium salt. All were dissolved to concentrations of 5-10 mM in a buffer containing 50 mM Tris-HCl, 1 mM EDTA and 25% D_2O. pH's (meter reading) were adjusted by adding small aliquots of 1 N HCl and 1 N NaOH, attempting to minimize dilution of the buffer during the titration. ^{31}P-NMR spectra were taken at 109.3 MHz on a Bruker HXS-270 spectrometer. Spectra were proton decoupled, pulses of 65° were used and the delays between individual pulses was 2 sec. In the course of our pH titrations of the model compounds we did not observe broadening of the resonances, indicating that the exchange rates between protonated and unprotonated species are all in the fast exchange limit.

A "non-linear least squares" computer program (written by R. Read) was used to calculate pKa's and "Hill coefficients" essentially as described by Markley [16] for the titration of histidine residues. Only fits which accounted for over 99% of the variance were accepted. Some of the fits were visually inspected by using a second program (courtesy of Dr. I. Carney) which plotted the calculated lines and experimental points.

Results and Discussion

Tropomyosin. This two stranded coiled-coil rod like protein of molecular weight 66,000 is involved in the calcium regulatory system of skeletal muscle [2]. It can form long filaments by aggregation of individual molecules involving an overlap of eight or nine amino acid residues at the NH_2- and COOH- terminal ends. A single phosphorylation site is located at the penultimate serine-283 residue. It has been proposed that this moiety can form salt linkages with lysine-6 and lysine-12 respectively and would thus stabilize the head to tail overlap [17]. Since only a small fraction of all molecules is phosphorylated, samples with high protein concentrations are required for the relatively insensitive ^{31}P-NMR measurements. Due to the above mentioned polymerization such samples are extremely viscous. At high ionic strength (1 M KCl) the polymerization is abolished and such samples have lost their gel-like appearance.

pH titration data obtained at high and low ionic strength are shown in Fig. 1. The comparison with the titration curve obtained for ovalbumin SerP-68 [18] shows that for both ionic strengths the titration behaviour is

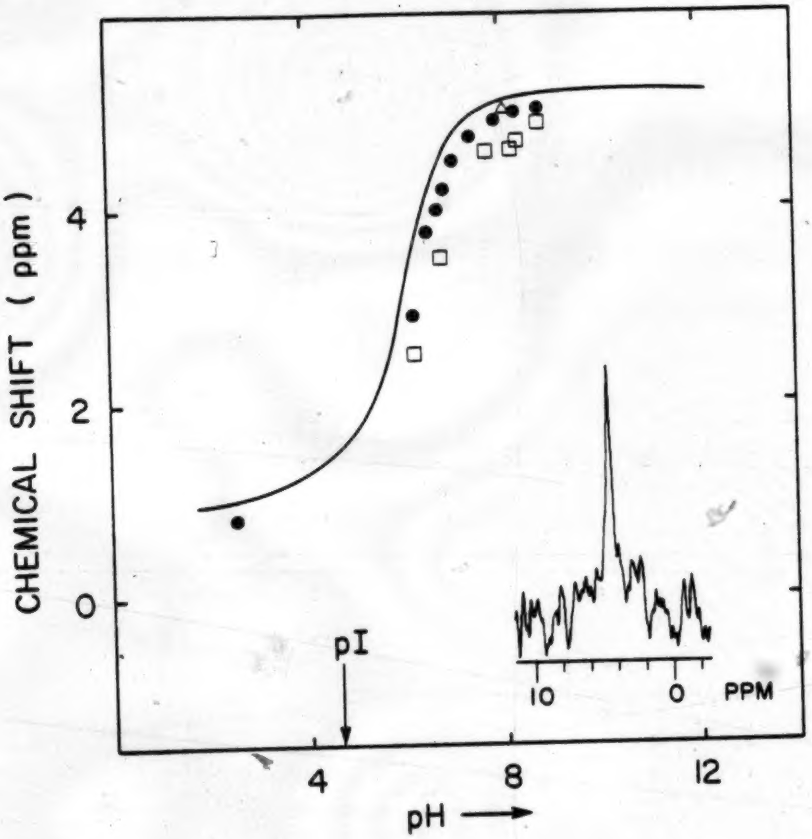

Fig. 8.1 ^{31}P-NMR (proton decoupled) pH titration of rabbit skeletal muscle tropomyosin ($\alpha\alpha$). The line drawn for comparison is that observed for serine phosphate-68 of ovalbumin [18]. For most spectra 25,000 scans of 50 mg/ml solutions were collected. The insert shows such a spectrum at pH 7.75 (no KCl) linebroadening 25 Hz. □, no additions, ●, addition of 1 MKCl, △, tropomyosin $\alpha\alpha$ and $\alpha\beta$ mixture + 1 MKCl, ratio of $\alpha:\beta$ = 3.5:1. Other conditions as described in Experimental Procedures.

similar to that of other phosproteins. A deprotonated phosphoserine standard gives rise to a resonance at 4.5 ppm in an ^{31}P-NMR spectrum. This is the same as we observed here for the polymerized form. The unpolymerized form has undergone a downfield shift of 0.3 ppm. This is most likely caused by the change in ionic strength, since the reasonance for the phosphoserine standard appears at 4.7 ppm in 1 M KCl. Small shifts due to differences in hydrogen bonding may make contributions as well [19]. The normal titration behaviour of the protein bound residue indicates that it is exposed to the solvent at least part of the time and can undergo protonation or deprotonation in the polymerized as well as unpolymerized state. Furthermore, we can conclude that at physiological pH the residues are mainly present in the dianion form.

The insert in Fig. 1 shows a representative spectrum of polymerised tropomyosin at pH 7.75 with a natural linewidth at half height of 20 Hz. All other spectra of polymerised tropomyosin had a linewidth of 25 ± 5 Hz. This narrow linewidth indicates considerable mobility for a residue in a polymer of this size [9]. This may seem to contradict the earlier suggestion [17] that the residue would be salt linked to lysines residues and thus rigidly held in the polymerized state. However, even in that case limited rotation of the phosphoryl moiety might still be possible, resulting in line narrowing. It is also possible that the observed narrow resonance is the fast-exchange averaged line originating from two conformations, one involving the salt linkage, the other one facing the solution. A ^{1}H NMR study of the tyrosine residues of tropomyosin has indicated that these have a high degree of motion and that the molecule is a very flexible rod in the polymerized as well as depolymerized state [20]. Although a higher degree of rigidity might have been expected for the head to tail overlap region, our data show that at least the penultimate serine phosphate residue has considerable flexibility in the polymer. In a subsequent section we will consider other evidence suggesting that the proposed salt linkages may indeed exist [17]. Linewidths measured for the phosphate resonance of the depolymerised molecule were usually 5 Hz. A few samples displayed larger linewidths, the reason for this is not obvious at present. The value of 5 Hz suggests considerable mobility for this residue in the depolymerised form [9]. However, one must be cautious in comparing the mobility for this rod-like molecule with that of globular proteins. It should be mentioned here that some of the preparations used were contaminated with small amounts of inorganic phosphate or nucleic acids, which could both be easily discerned in the spectra by their different titration behaviour. Some of the inorganic phosphate originated from the serine-phosphate residue which appeared to be unusually labile to base. Similar contaminants were observed in ^{31}P-NMR spectra of preparations of myosin light chains [15].

Glycogen phosphorylase a. Phosphorylation of the serine-14 residue by phosphorylase kinase dramatically increases the activity of the enzyme [21]. X-ray studies of crystals grown in the presence of glucose have indicated that the phosphoryl moiety is hydrogen bonded to Arg43' of the neighbouring subunit and to Arg69 which is part of an α-helix that runs through the molecule to the AMP activator site [21]. One problem for ^{31}P-NMR studies of phosphorylase *a* is that the resonance for the catalytic pyridoxal-phosphate overlaps with that of the regulatory serine-phosphate. However, upon binding of the inhibitor glucose to the active site, the pyridoxal phosphate resonance is shifted upfield, virtually eliminating the overlap [22,23]. Interestingly, addition of glucose stimulates the action of the protein phosphatase on the enzyme [24]. It has been suggested that it may expel the serine-phosphate from its binding pocket and in doing so it becomes available to the phosphatase [24]. Consistent with this are ^{31}P-NMR measurements [22,23] that have shown that the residue has a higher mobility in the presence of glucose, while addition of activator AMP results in reduced mobility.

Our pH titration data are shown in Fig. 2. Due to instability and insolubility of the enzyme only a narrow pH range could be studied (pH 6.3-8.5). The comparison with the SerP-68 residue of ovalbumin indicates however that a normal titration curve can be drawn which levels around 4.2 ppm.

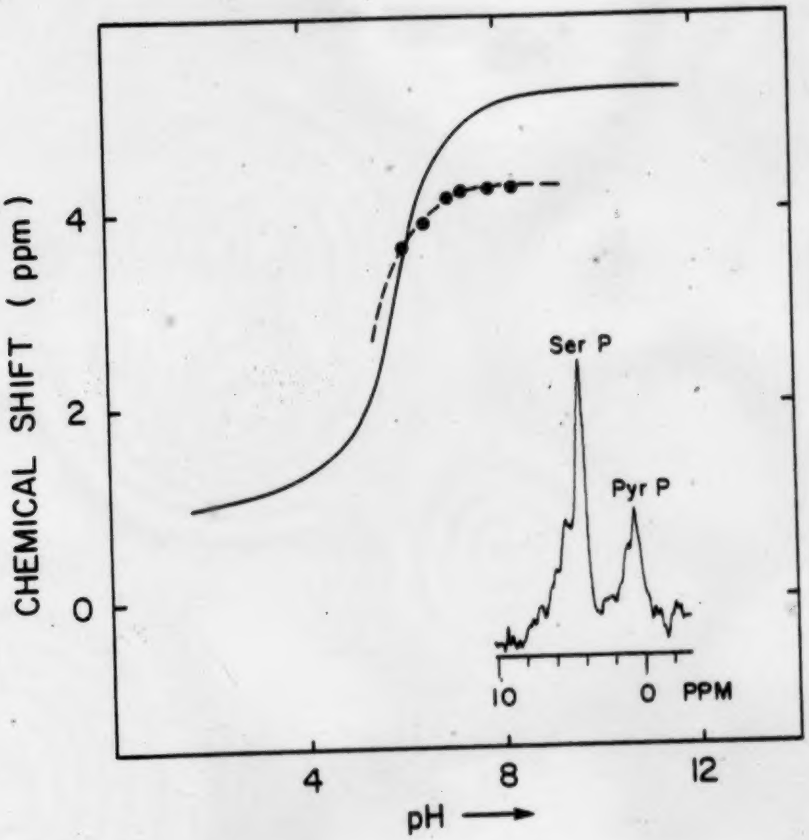

Fig. 8.2 Partial ^{31}P-NMR (proton decoupled) pH titration of rabbit skeletal muscle glycogen phosphorylase. For comparison the titration of the serine phosphate-68 of ovalbumin is shown [18]. The insert shows a spectrum at 25 mg/ml, pH = 7.3 linebroadening 25 Hz. 15,000 scans. Other conditions as described in Experimental Procedures.

This figure differs from the 4.5 ppm found for a standard phosphoserine solution, and this might possibly indicate hydrogen bonding of the serine-phosphate moiety [19]. The titration behaviour indicates however that the residue must be exposed to the solvent, at least part of the time. The insert in Fig. 2 shows the spectrum at pH 7.3 (70 ± 5 Hz). Linewidths measured at pH 8.2 and 6.3 were 60 Hz and 105 Hz respectively; thus the linewidth increases drastically with a decrease in pH. Elsewhere [9] we calculated on the basis of frequency dependent studies of the linewidths that the observed resonance in the presence of saturating amounts of glucose is the fast exchange average of not less than two conformations. Protonation of the serine-phosphate residue slows down the exchange between these two conformations, in keeping with the present results.[1] [2] This can be readily understood in terms of the scheme shown in Fig. 3. At high pH the two conformations as shown on the left are in fast exchange. At lower pH the conformation facing the solvent can become protonated. This will reduce the charge on the phosphoryl moiety, thereby reducing the strength of interaction of the arginines for this residue. The exchange

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[1] It could be argued that the protonation step itself is responsible for the observed increase in exchange broadening. We think this is very unlikely since for all model compounds studied fast exchange conditions prevailed.

[2] The increase in linewidth could be caused by a pH dependent conformational change instead of protonation. We feel this possibility can be excluded, since for the thiophosphoryl residue (with a pKa far below that of phosphoserine) a different dependency of linewidth and pH was observed [22], albeit in a different buffer and at a different spectrometer frequency.

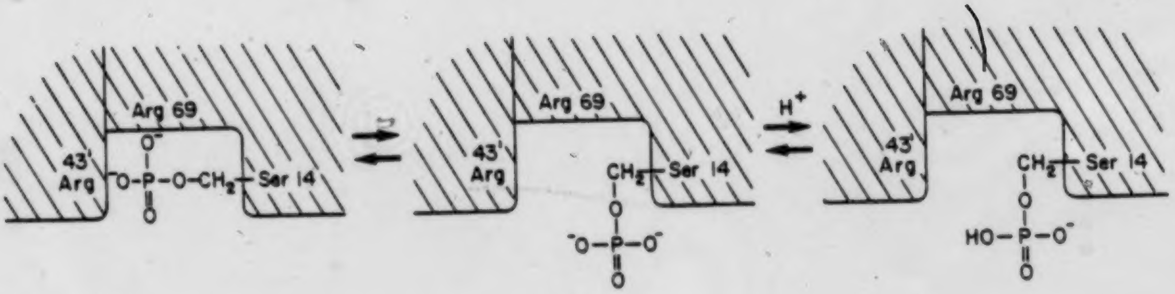

Fig. 8.3 Model for the serine phosphate site of glycogen phosphorylase *a* (in the presence of glucose) with essentially two conformations. The first one (on the left) is hydrogen bonded as observed in the crystal [21] and the second one is facing the solvent as deduced from its titratability (Fig. 2). This second conformation can become protonated as indicated on the right. For further explanation see text.

rate between the two conformations therefore drops, leading to a broader resonance. Thus in the absence of glucose and especially in the presence of AMP the equilibrium favors the form with the phosphoryl residue immobilised by the arginine residues as observed in the crystal and as indicated by ^{31}P-NMR measurement of the thiophosphoseryl moiety [22,23]. Binding of the inhibitor glucose to the active site shifts the equilibrium to the more mobile solvent exposed conformation. This model of allosteric regulation of the protein substrate of the protein phosphatase is consistent with the kinetic data of Detwiler *et al*. [24] showing that addition of glucose activates the phosphatase and addition of AMP inhibits its action.

Model compounds. Figures 4 and 5 show the pH-titration curves for the pKa$_2$ of a wide variety of phosphorus containing model compounds. We have not attempted to study changes in the pKa$_1$ since these are usually not accompanied by readily detectable changes in the chemical shifts as has been shown for the titration of phosphodiesters [10,12] or inorganic phosphate [13]. In the course of our titration studies we noticed that the phosphomonoester compounds were slightly unstable at high pH, the phosphoramidates were moderately unstable at low pH, and the acyl-phosphates are very unstable (up to 50% hydrolysis in 20 min.) at both pH extremes. These results agree with other reports [6]. Breakdown would have affected normal pH titrations, but in these

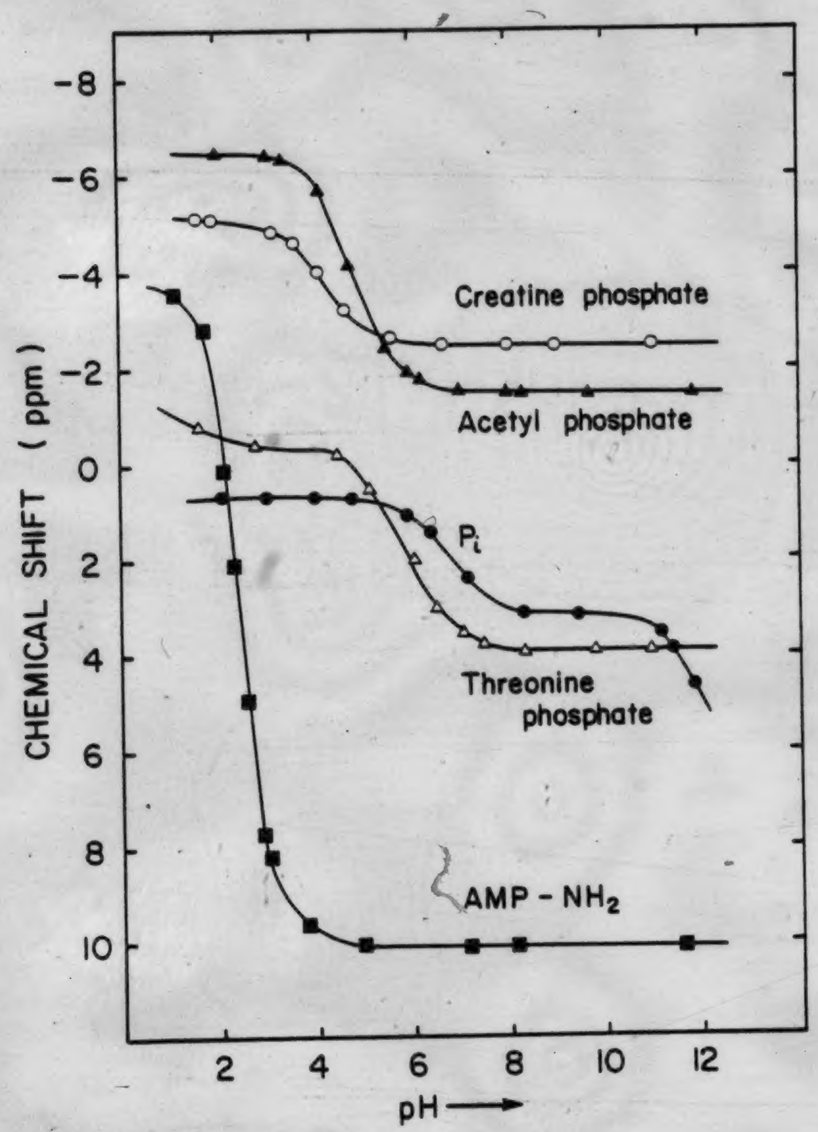

Fig. 8.4 ^{31}P-NMR (proton decoupled) pH titration of 5-10 mM solutions of a series of phosphorus containing model compounds. Other conditions were as described in the text.

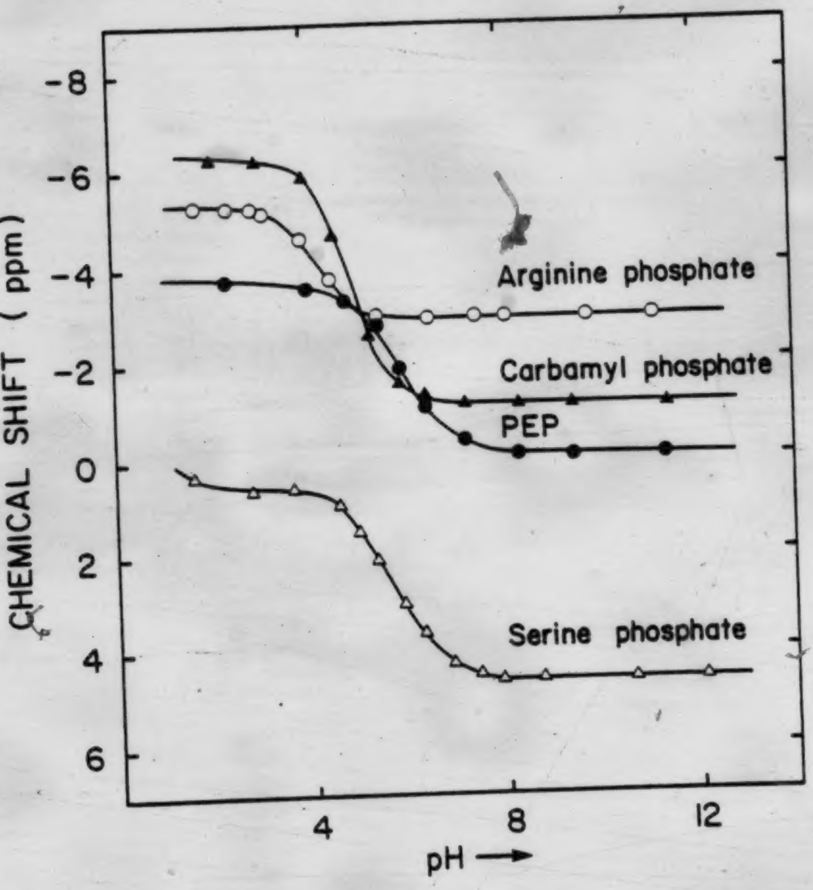

Fig. 8.5 ^{31}P-NMR (proton decoupled) pH titration of 5-10 mM solutions of a series of phosphorus containing model compounds. Other conditions were as described in the text.

experiments the resonances seen for the compound and its breakdown products are well separated and can be studied independently. The usual breakdown product observed was inorganic phosphate, but in the case of the AMP-5'-phosphoramidate a resonance corresponding to AMP was observed as well.

A comparison of the data in Fig. 4 and 5 shows that for compounds with identically substituted phosphoryl moiety similar curves are obtained. For example, serine- and threonine-phosphate, or acetyl- and car[illegible]myl-phosphate, behave quite similar. This becomes even more obvious from the data collected in Table 8.1. Not only are the chemical shifts (δ) related, but also the change in chemical shift over the pKa, titration ($\Delta\delta$) is virtually the same. Most of the compounds studied have other charged groups that could possibly interact with the phosphoryl moiety. Apparently these groups do not have overriding effects on the chemical shifts, consistent with other reports showing that hydrogen bonds give rise to only minor changes in the chemical shift [19].

Some dissimilarities exist within the class of phosphoramidates (see Table 8.1. Arginine- and creatine-phosphate are very similar, but different shifts are observed for the phosphohistidines. This is possibly due to ring current shifts originating from the imidazole ring; similarly the chemical shift observed for 5'-adenyl-O-

Table 8.1

Chemical Shift Parameters Measured for Various Phosphorus Model Compounds

	Deprotonated δ (ppm)	$\Delta\delta$ (ppm)
Orthophosphate	3.2	2.5
Phosphomonoesters		
- serine-phosphate	4.6	4.1
- threonine-phosphate	4.0	4.5
- pyridoxal-phosphate (a)	3.7	3.8
- pyridoxamine-phosphate (a)	3.7	3.6
- phosphoenolpyruvate	0.0	3.7
Phosphoramidates		
- arginine-phosphate	-3.0	2.4
- creatine-phosphate	-2.5	2.6
- N-3 phosphohistidine (b)	-4.5	0
- N-1 phosphohistidine (b)	-5.5	0
- phosphoramidate (b)	9.0	11.5
- AMP-5'-phosphoramidate	10.0	13.7
Acyl phosphates		
- acetyl-phosphate	-1.5	5.0
- carbamyl-phosphate	-1.1	5.2

(a) Data taken from Ref. 25.

(b) Data taken from Ref. 13.

tyrosine is different from that of other phosphodiesters [8,10,12]. It is not clear why no pKa_2 is measureable for the phosphohistidine residues. Small inflections have been recorded, but these were assigned to other titrating groups (for discussion see 9,13). The very large shifts seen upon deprotonation for the two phosphoramidates are noteworthy. Both have a protonatable $-NH_2$ group and this titration may reflect its deprotonation. Note that this assignment is different from that earlier proposed [13], but is in agreement with other interpretations [26]. A possible explanation for the large $\Delta\delta$ is that such compounds can form stable zwitterions [26] and such structures will presumably have different bond angles of the substituents around the central phosphorus atom. Changes in bond angle lead to large differences in chemical shift [27].

Table 8.2 summarizes the pKa's and Hill coefficients [16] we determined for model compounds and a few phosphoproteins. The data show that within one group of phosphoryl compounds the pKa's remain relatively the same. Thus the presence of charged side chains (or 1 M KCl) does not result in obvious changes. The pKa's determined also show that all phosphoryl groups are dianionic at physiological pH. The values determined for the phosphoproteins seem generally somewhat higher than those observed for the phosphomonoesters free in solution, but this behaviour is not consistent (see Table 8.2). Most intriguing is the pattern that

Table 8.2

Comparison of pKa's and Hill Coefficients

	pKa	Hill Coefficient
Model Compounds		
- orthophosphate	6.80	0.95
- orthophosphate + 1 MKCl	6.74	0.98
- serine-phosphate	5.75	0.92
- serine-phosphate + 1 MKCl	5.85	0.93
- threonine-phosphate	5.95	0.97
- phosphoenolpyruvate	5.89	0.91
- arginine-phosphate	4.26	1.16
- creatine-phosphate	4.21	1.09
- acetyl-phosphate	4.79	0.91
- carbamyl-phosphate	4.91	1.11
Phosphoproteins		
- ovalbumin (a) Ser-P 68	6.00	0.94
Ser-P 344	6.04	0.87
- phosvitin (b)	5.79	0.70
	6.17	0.71
	5.35	0.76
- tropomyosin no KCl	6.23	1.35
+ 1 MKCl	6.12	1.03
- salivary proteins (c)	6.51	-
(human)	6.56	-

(a) See Chapter 9.

(b) See Chapter 11. The data represent the titration date observed for three different groups of the total 125 serine phosphate residues, that can be resolved in an ^{31}P-NMR spectrum.

(c) See Ref. 30. The Hill coefficients were between 0.92 and 1.07 but it was not specified to what resonance they corresponded.

emerges from the calculated Hill-coefficients (n). (For comparison of the structures see Fig. 6). For most model compounds as well as for the two ovalbumin serine-phosphates the value is between 0.87-0.98. Obviously n is not equal to unity and this may be a reflection of the presence of the other negative charge (pKa$_1$) on the phosphoryl moiety. A similar range of n values has been reported for studies on ATP analogs [28]. The serine-phosphates of phosvitin, however, all have values below 0.76. The anti-cooperativity of these titrations ($n<<1$) indicates the presence of other groups in the vicinity that titrate in the same pH region [16]. This outcome was therefore not unexpected since 60% of the amino acids in this protein are serine-phosphate residues [29]. Thus proximal negative charges lead to a decreased Hill coefficient. Similarly, proximal positive charges may lead to $n>1$, as indicated by the effect of the positively charged guanidinium group on the Hill coefficients determined for creatine- and arginine-phosphate and the differences in n determined for carbamyl- and acetyl-phosphate.

This empirical correlation between n and charge allows us to interpret the values obtained for tropomyosin (Table 8.2). At low ionic strength the protein is polymerized. As indicated by the $n = 1.35$, positive charges must be close to the phosphoryl moiety. This gives support to the proposed [17] salt linkages between the dianionic phosphoryl moiety

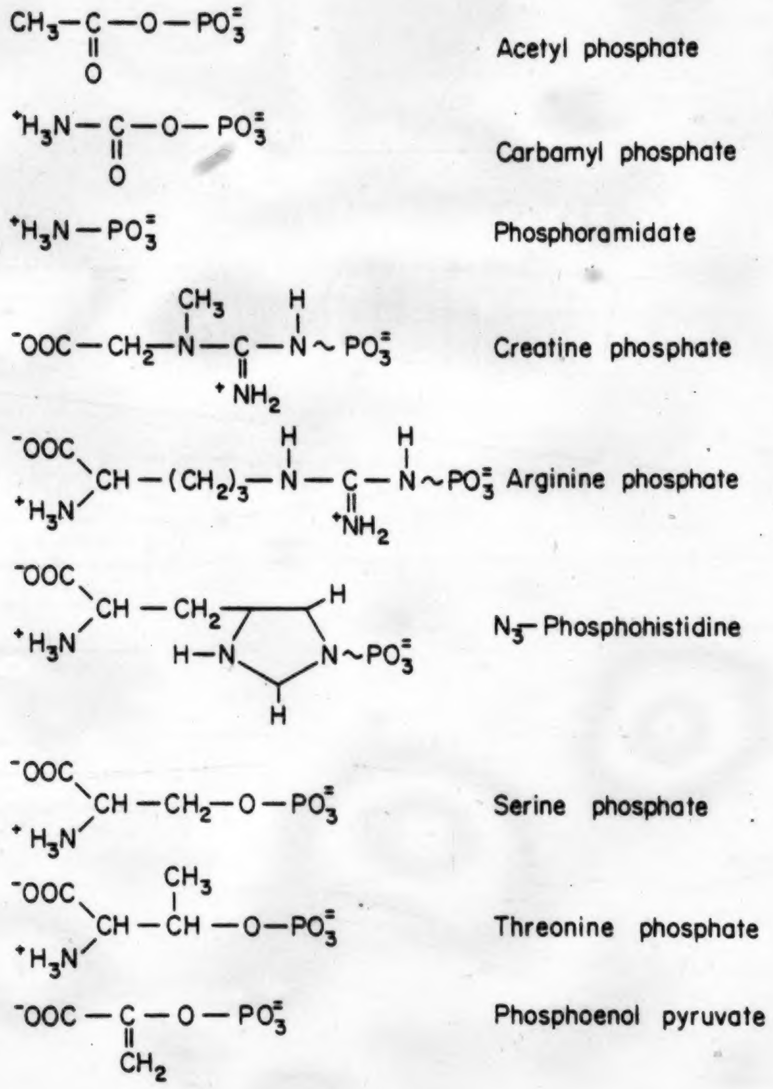
Acetyl phosphate
Carbamyl phosphate
Phosphoramidate
Creatine phosphate
Arginine phosphate
N3- Phosphohistidine
Serine phosphate
Threonine phosphate
Phosphoenol pyruvate

Fig. 8.6 Structures of the model compounds used to obtain the data of Tables 8.1 and 8.2, and Figs. 8.4 and 8.5.

and the positively charged lysine 6 and 12 residues in the head to tail overlap region. At high ionic strength this interaction is broken as indicated by the decreased $n = 1.03$, which now approximates values observed for phosphomonoesters ($n = 0.95$). Further studies with model compounds, phosphopeptides and phosphoproteins are necessary to establish if the empirical correlation as observed here will be a general one. If so, it could be used in future studies to probe the environments of titratable phosphoryl moieties in phosphoproteins as demonstrated above for tropomyosin.

Conclusions

Since the chemical shift measured in an ^{31}P-NMR spectrum is a complex function of many variables it is not the most reliable parameter to measure the protonation state of a protein-bound residue [8,28]. We have shown here by means of pH titration for both tropomyosin and glycogen phosphorylase *a* (in the presence of the inhibitor glucose), that both residues are dianionic (see also ref. 9) and that both residues are at least part of the time exposed to solvent since they shift with pH. This seems to be a general phenomenon for regulatory sites. Similar studies on the regulatory serine phosphates of ovalbumin [18], troponin T [14] and myosin light chains [15] have given equivalent results. In contrast, active site residues or tightly bound nucleotides in general do not shift with pH [8]. Moreover for all regulatory sites studied so far, considerable flexibility has been implied either by mobility or by exchange between two conformations. We suggest that this flexibility may be necessary for the protein-kinases and -phosphatases to interact with their substrate proteins. In keeping with this generalization, active site residues are generally immobilized on the protein [9].

Two different models have been proposed to explain how regulatory phosphorylation sites may exert their function. In the first model the dianionic serine-phosphate residue is salt-linked to either arginine or lysine residues [17,21,22] and in doing so may help stabilise one specific conformation. In the other model the mobile phosphoryl group may prevent an interaction between protein domains [14,15]. Our preliminary characterizations of the titration behaviour by means of the Hill coefficient may allow for distinguishing between these two different functions. As we have shown here only this entity and not the chemical shift (δ), or the change in chemical shift ($\Delta\delta$) or the pKa_2 seems to be uniquely altered by proximal charged entities.

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CHAPTER 9

^{31}P-NMR STUDIES OF HEN EGG-WHITE OVALBUMIN

Summary

Ovalbumin contains two serine phosphate residues that give rise to two well resolved resonances in an ^{31}P-NMR spectrum. Ovalbumin samples that have been digested with a variety of phosphatases give rise to only one serine phosphate resonance, indicating that one of the two phosphorylated sites is relatively inaccessible for phosphatase action. By comparison of the amino acid sequence of the peptide containing the susceptible phosphate to the overall primary structure we have been able to assign the resonance observed (at pH 8.3) at 5.0 ppm to SerP68 and the resonance observed at 4.75 ppm to SerP344. Both phosphoryl moieties are surface residues as inferred from pH titration experiments and their susceptibility to phosphatases. Both residues have a pKa = 6.00-6.04. Analysis of the Hill coefficients measured for the pH titrations and the *JPH* coupling constants indicate that neither residue interacts with other charged groups on the surface of the protein. We have also performed frequency dependent experiments using five different NMR spectrometers. At higher magnetic field strengths the contribution of chemical shift anisotropy to the linewidth becomes very significant. We have calculated from the field dependent terms that SerP344 is mobile with

respect to the protein surface but that SerP68 is more restricted in its motion. This last residue is also involved in a pH dependent conformational change, since it is shielded from hydrolysis by phosphatases at higher pH. A comparison of the amino acid sequence of the SerP68 site shows that it has a striking homology to the active site peptides of a wide variety of hydrolytic enzymes. Moreover, a comparison with the primary sequences of caseins suggest that both proteins are phosphorylated by a protein kinase that specifically recognizes a Ser-X-Glu peptide.

Introduction

Ovalbumin is the most abundant protein of egg-white. It can be readily purified and crystallized in large quantities (Hofmeister, 1889). Although its properties have been frequently studied as a "model protein" very little is known about its biological function (Taborsky, 1976). The protein is synthesized in the chick oviduct before it is deposited in the egg. *De novo* synthesis is under hormonal control and requires the presence of steroid sex hormones. This control has been extensively studied at the level of transcription as well as translation (Palmiter, 1974). Moreover the protein has also provided interesting insights for students of eukaryotic gene organisation (Breathnach *et al*., 1977). The complete amino acid sequence is known at present, deduced both from the mRNA sequence (McReynolds *et al*., 1978) and by conventional amino acid sequencing techniques (Nisbet *et al.*, 1981). The protein is a monomer comprised of 385 residues with a molecular weight for the polypeptide chain of 43,000. In contrast to most other proteins that are translocated across a membrane ovalbumin does not possess a hydrophobic N-terminal signal sequence, but is believed to contain an internal signal sequence to facilitate its secretion (Lingappa *et al.*, 1979). Most preparations of the enzyme display some form of heterogeneity, some of which arise from the genetic variants of the protein. The two variants that have been characterized have undergone only one base change resulting in a replacement between an acid and its amide (Nisbet *et al.*, 1981; Ishihara *et al.*, 1981). Other contributions to the heterogeneity mainly arise from differences in the level of post-translational modifications, such as the N-acetylation of the N-terminal amino acid (Narita *et al.*, 1968) or the glycosylation of the Asparagine 292 residue. Inspection of the amino acid sequence reveals that the latter site has the Asn-X-Ser/Thr sequence typical to all N-glycosylated sites (Aubert *et al.*, 1981). Interestingly, ovalbumin contains a second region with identical sequence which is never glycosylated *in vivo* but can be modified *in vitro* (Glabe *et al.*, 1981). The *in vivo* heterogeneity arises mainly from the different oligosaccharides that can be attached to Asn292 (Tai *et al.*, 1977; Atkinson *et al.*, 1981; Ishihara *et al.*, 1981); even when eggs from a single hen are tested the oligosaccharides are still hetereogeneous (Iwase *et al.*, 1981).

The third post-translational modification is the phosphorylation of serine residues 68 and 344 (Nisbet *et al.*, 1981). Although it has been recognized since the turn of the century that ovalbumin is a phosphoprotein (Osborne & Campbell, 1900), very little is known about the reactions leading to phosphorylation or dephosphorylation of the protein. Heterogeneity in the electrophoretic behaviour of ovalbumin preparations has been attributed to a different extent of phosphorylation (Linderstrøm-Lang and Ottesen, 1949). Enzymatic phosphatase digestions as well as amino acid determinations of peptides resulting from a partial acid hydrolysis of the protein indicated the presence of two nonoverlapping serine phosphate sites (Flavin, 1954; Perlmann, 1955). These observations were confirmed more recently by the peptide sequencing studies of Milstein (1968) and by the completion of the determination of the overall primary structure (Nisbet *et al.*, 1981).

Studies with minced oviduct preparations have shown that the dephosphoenzyme is the first product formed which is in turn phosphorylated to the bisphosphorylated enzyme (Sanger & Hocquard, 1962). However, others have been unable to observe the bisphosphorylated enzyme in similar preparations (Carey, 1966). This discrepancy was attributed to a different hormonal status of the preparations used in both experiments (Carey, 1966) raising the question of a protein kinase possibly under the control of steroid hormones.

Disappointingly little is known about the fate of the protein-bound phosphorus in the egg. It has been demonstrated that dephospho-ovalbumin appears in the yolk of fertile egg after the growth of the embryo has begun (Saito *et al.*, 1965).

Here we will report on ^{31}P-NMR studies of hen egg white ovalbumin. The two serine phosphate residues give rise to resonances that are well resolved in an ^{31}P-NMR spectrum and that could be readily assigned. Moreover we have characterised both residues in terms of metal binding properties, mobility, titratability, environment and stability towards digestion by a variety of phosphatases.

Experimental procedures

Ovalbumin was obtained from Sigma Chemical Company. A variety of batches of different purity (V, VI) were used but no differences were observed for these preparations in ^{31}P-, ^{1}H- and ^{13}C-NMR spectra. Moreover all batches were judged pure by behaviour as one band on SDS-acrylamide gel electrophoresis (see Chapter 12). All other enzymes used were purchased from the same source with the exception of TPCK-trypsin which was obtained from Worthington. All these preparations were used without further purification. Phenylmethyl sulfonyl fluoride (PMSF) was obtained from Calbiochem. Ultra pure urea and Tris were purchased from Schwarz-Mann. All other chemicals were analytical grade. The pH was measured on a Radiometer PHM62 pH meter equipped with a glass electrode. The buffers that were used contained 50 mM Tris-HCl, 1 mM EDTA and 25% D_2O (Biorad 99.8%) at pH 8.3. NMR samples were 1.5 ml in 10 mM precision tubes (Wilmad) fitted with teflon Vortex-plugs. Most NMR measurements were performed at 28°C on a Bruker HXS-270 spectrometer at 109.3 MHz. Typical conditions for the experiment were a 60° flip angle and a 2 sec recycle time. Proton decoupling during acquisition was used unless otherwise indicated in the text. Frequency dependent experiments were performed at five different frequencies. Spectrometers used were as described in Chapter 4, and in addition the spectra at 145.7 MHz were obtained at the Bruker HX-360 at the Dutch National NMR facility at the State University of Groningen, Groningen, The Netherlands. The linewidths were measured from the resonances at half height and were corrected for the additional linebroadening introduced by the computer digital filtering. The nuclear overhauser enhancements were measured by determining the difference between spectra that were proton-decoupled only during acquisition or that were decoupled during the whole cycle.

Results

Assignment of the resonances. Figure 1a shows a proton-decoupled ^{31}P-NMR spectrum at 109.3 MHz of a 1 mM solution of ovalbumin at pH 8.3. Similar spectra were obtained over a wide variety of concentrations (0.1-2.5 mM).

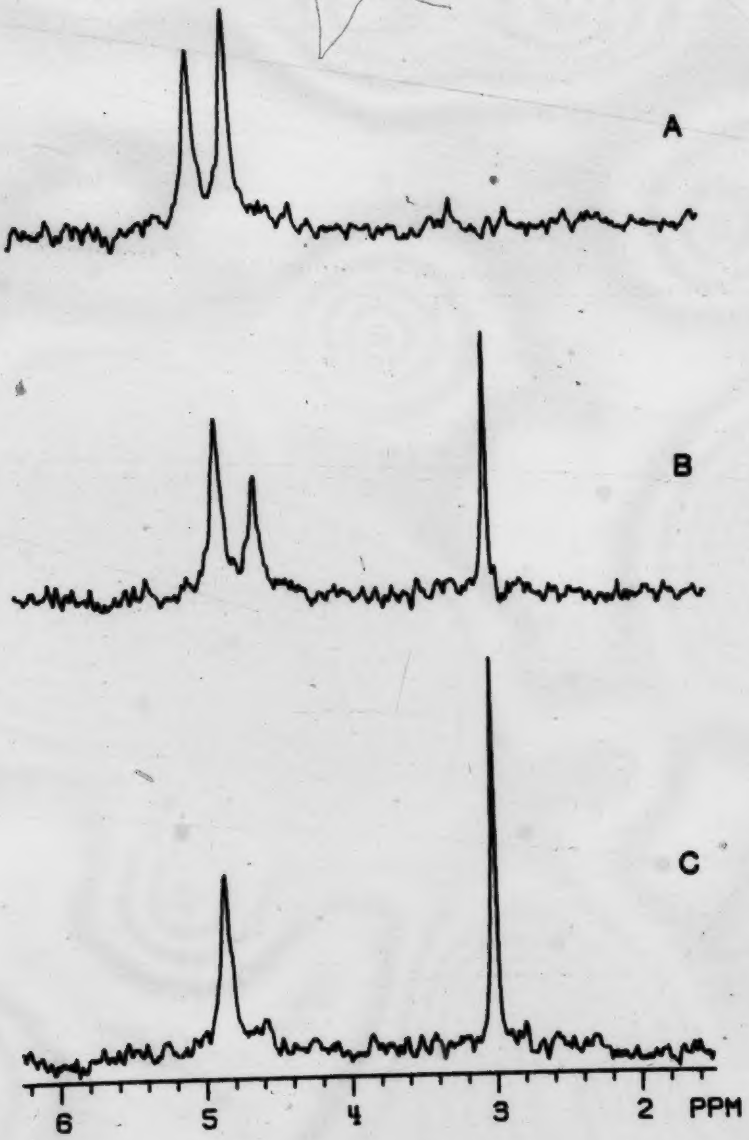

Fig. 9.1 Proton-decoupled ^{31}P-NMR spectra at 109.3 MHz of hen egg-white ovalbumin. (a) concentration of ovalbumin was 1.0 mM, in 50 mM Tris-HCl, 1 mM EDTA pH 8.3; 500 acquisitions, additional line-broadening 2.5 Hz; (b) same as in a; 1 hour after the addition of 15 mM $MgCl_2$ and 15 units *E. coli* alkaline phosphatase. The upfield shift of both resonances with respect to those observed in spectrum 1a is caused by the addition of metal ion; (c) same as in b; 20 hours after the addition of *E. coli* alkaline phosphatase.

Two well resolved resonances can be seen at chemical shift positions of 5.00 and 4.75 ppm respectively (Bock & Sheard, 1975). Integration indicated that the two resonances are of equal intensity. Thus each resonance represents one serine phosphate residue of the bisphospho protein. Early studies had indicated that ovalbumin containing varying amounts of phosphates behaved differently upon electrophoresis and could be separated in the bis-, mono- and dephosphorylated forms (Linderstrøm-Lang and Ottesen, 1949). Subsequent experiments with several phosphatase preparations have shown that one equivalent of phosphate could be readily removed to form the monophosphoprotein. Removal of a second equivalent required prolonged incubations (Perlmann, 1955). Figures 1a and 1c show a sample of ovalbumin digested with *E. coli* alkaline phosphatase at pH 8.75. The residue corresponding to the resonance originally at 5.0 ppm was resistant to degradation. The other resonance at 4.75 ppm has disappeared, with the appearance of a new peak at 3.0 ppm, which could be assigned in terms of chemical shift and pH titratability to inorganic phosphate. Experiments with a variety of other phosphatases (see next section) have confirmed that the SerP resonance on the left is always less susceptible to digestion. Thus the one equivalent of phosphate that is removed (Perlmann, 1955) all originates from one residue. Studies of partial acid hydrolysates have suggested that the phosphatase-sensitive serine phosphate is present in the peptide sequence SerP-Ala and that the phosphatase-resistant phosphate occurs in a sequence Asp-SerP-Glu-Ile-Ala (Flavin, 1954).[1] By comparing these to the overall sequence we have assigned the resonance at 5.00 ppm to SerP68 and the resonance at 4.75 ppm to SerP344.

Susceptibility to phosphatase digestion. At the outset of this study, we performed the digestions with alkaline phosphatases (*E. coli* and calf intestine) at pH 8.75 and with the acid phosphatases at pH 5.5. Thus the enzymes were incubated close to their respective pH optima, at pH's were ovalbumin is stable. The results (see Table 1) suggested to us at that time that SerP344 was a good substrate for all phosphatases, but that SerP68 could be readily digested only by acid phosphatases. This would have been an indication of different specificities for different phosphatases (Neuman, 1968). However, as the other results in Table 1 show, SerP68 can be digested at lower pH by alkaline phosphatase and acid phosphatases become less efficient in hydrolysing the phosphate of residue 68 at higher pH. These observations suggest that the substrate ovalbumin may undergo a pH-dependent conformational change and that residue SerP68 is shielded at higher pH. To substantiate this shielding effect further we reasoned that peptides, in contrast to the native protein, should be susceptible to *E. coli* alkaline phosphatase digestion at pH 8.75. Thus in a subsequent experiment we

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[1] Note that in later studies the sequence of the second peptide has been corrected to Asp-SerP-Ile-Glu-Ala. (See Milstein, 1968; Taborsky, 1974; McReynolds *et al*., 1978 and Nisbet *et al*., 1981).

Table 9.1

Percentage degradation of the two phosphoserine residues of ovalbumin by different phosphatases

pH		Alkaline Phosphatase *E. coli*	 Calf Intest.	Acid Phosphatase Potato	 Wheat Germ
pH 8.75	SerP68	10	30	0	0
	SerP344	100	100	0	20
pH 7.0	SerP68	25	75	75	90
	SerP344	100	100	100	100
pH 5.5	SerP68	75	90	100	100
	SerP344	100	100	100	100

All experiments were performed by incubating 1 mM ovalbumin with 20 units of phosphatase for 20 hours at 37°C. Buffers contained 15 mM Mg^{2+} and either 50 mM Tris/HCl (pH 8.75), or 50 mM Mes (pH 5.5) or a mixture of both buffers (pH 7.0).

subjected 1 mM ovalbumin samples to proteolytic cleavage by trypsin, chymotrypsin, subtilisin and pronase (pH 7.5, 4% protease, 10 hours incubation at 37°C). The resulting mixtures were analysed by ^{31}P-NMR spectroscopy and SDS-acrylamide gel electrophoresis. Trypsin was found to be ineffective in degrading the protein, consistent with previous reports (Taborsky, 1974), but the other proteases degraded the protein to small peptides. Pronase is apparently contaminated with a phosphatase, since in this sample the resonances of the two serine-phosphate residues had disappeared and only resonance corresponding to inorganic phosphate was observed in the ^{31}P-NMR spectrum. Thus both the chymotrypsin- and subtilisin-digested samples, as well as a control that had been incubated without protease, were treated with 0.5 mM PMSF to inactivate residual protease activity. These samples were then stored at 4°C for 20 days in order to inactivate the PMSF since compounds such as PMSF may affect activity of phosphatases (Greenberg & Nachmansohn, 1965). Finally the samples were digested with *E. coli* alkaline phosphatase at pH 8.75 (conditions as in Table 1). The resulting ^{31}P-NMR spectra showed that in the protease treated preparations the SerP68 as well as the SerP344 were susceptible to phosphatase digestion, but in the control only SerP344 was susceptible. This is in agreement with results on peptides by others (Milstein, 1968). Thus all of these data are in support of the idea that on ovalbumin SerP68 is shielded at high pH and that it becomes deshielded at low pH.

We also studied the progress curve of the *E. coli* alkaline phosphatase digestion of SerP344 at pH 8.5 by following the decrease in the SerP344 resonance and the increase in the Pi resonance. The data indicated strong product inhibition, consistent with the model presented for this enzyme (Hull *et al.*, 1976). The progress curve of potato phosphatase digestion of ovalbumin at pH 5.5 did not indicate any product inhibition but clearly showed that hydrolysis of SerP344 is much faster than that of SerP68. Thus although SerP68 becomes more deshielded at low pH it is still not a favoured substrate of the phosphatases. A possible explanation could be that the combination of the serine phosphate and the carboxylate of the adjacent Asp residue simulates compounds such as PEP, carboxyphenylphosphate and L-phosphomandelic acid which are known to give rise to less active enzyme substrate complexes (Reid & Wilson, 1971). It has also been suggested that a possible difference in protonation state of the phosphoryl moiety could account for a difference in substrate specificities for phosphatases (Neuman, 1968). However, the results presented in the next section show that the residues have virtually indistinguishable pH titration behaviour, rendering this an unlikely explanation for the differential phosphatase sensitivity of residues SerP68 and SerP344.

pH dependence. Figure 2 shows the pH dependence of both ovalbumin Ser-P residues. Obviously both residues are exposed to solvent. No changes in linewidth were observed in the course of the titration, thus protonated and deprotonated species are in fast exchange on the NMR time scale. Such behaviour is normal for a variety of model-compounds (Chapter 8), but it does not have to be a phenomenon general to all phosphoproteins, as was indicated by a split ^{31}P-NMR resonance observed at pH's near the pKa in a titration study of human salivary phosphoproteins (Bennick *et al.*, 1981). This last result may be an indication of slow or intermediate exchange between protonated and deprotonated species. The pKa's and Hill coefficients (n) that we have calculated for the two phosphoserines 68 and 344 are pKa = 6.00 and 6.04 and n = 0.94 and 0.87 respectively. As indicated in Chapter 8, these data are typical for a phosphoprotein and they indicate that no charged groups are located proximal to the phosphoryl moiety. In the course of these studies we have exposed ovalbumin to pH 12.5 at 22°C. After 15 hours both SerP's had been completely hydrolysed. The rate of disappearance of SerP344 was higher than that of SerP68. This observation is consistent with previous reports (Perlmann, 1955).

One possible explanation for the differences observed in the chemical shifts of the protein bound residue with respect to phosphoserine standard (4.5 ppm, pH 8.3) is that

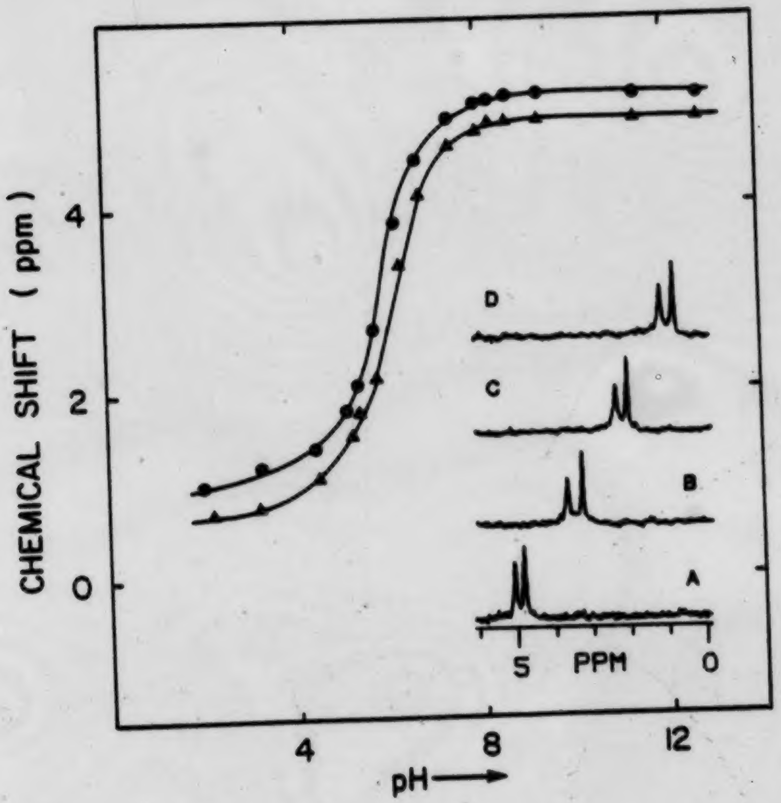

Fig. 9.2 ^{31}P-NMR pH titration of ovalbumin (1 mM). the spectra shown in the insert were obtained at (a) pH 8.3; (b) pH 6.2; (c) pH 5.6; (d) pH 3.3. The following symbols are used (•) SerP68 (▲) SerP344. Other conditions were as described in Figure 1.

they arise from the tertiary structure of the protein. However the presence of 8 M urea and 25 mM DTT did not result in any obvious changes in the spectrum observed at pH 8.3. Control experiments with CD and ^{1}H-NMR confirmed that the protein was totally unfolded under these conditions, although these studies were performed at a lower protein concentration (data not shown). We also performed a pH titration experiment under these denaturing conditions. As the results of Fig. 3 indicate, very similar titration curves are obtained for both residues. Although the residues move closer together at certain pH's than they do in the native protein they never actually overlap and they remain easily recognisable in the spectra. It is noteworthy that SerP68 had a linewidth about equal to that of SerP344 at pH 8.3 indicating that this residue is now less restricted in its motions than it was in the native protein. However the linewidths are such that both residues do not yet resemble a phosphoserine standard. Possibly the area of the protein in which both phosphates are located is held together by charge interactions that remain intact in 8 M urea. Such structures may exist in ovalbumin (Taborsky, 1974). The pKa's deduced from Fig. 3 are 6.61 and 6.56 for residue 68 and 344 respectively. These are higher than the ones we have measured for the native protein. This may be a reflection of the uncertainty of pH measurement under these conditions. For example the pKa's of a phosphoserine and Pi were 6.10 and 7.40 when 8 M urea was present, but they

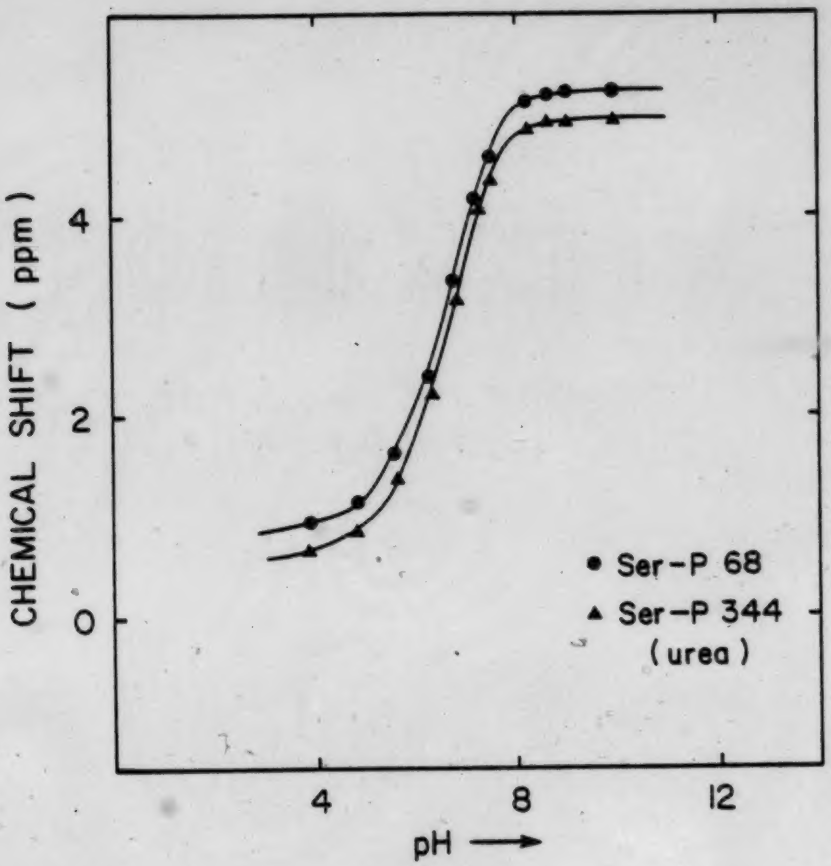

Fig. 9.3 pH titration of ovalbumin (1 mM) in 50 mM Tris, 1 mM EDTA, 8 M urea and 25 mM DTT. Other conditions were as described in Figure 2.

measured at 5.75 and 6.80 in the absence of the denaturant.

Magnesium titrations. One of the functions suggested for ovalbumin is for storage of metal ions (Taborsky, 1974). If the two phosphates are an integral part of a metal ion binding site they would be expected to display a change in chemical shift upon binding of metal ions. Such behaviour has been demonstrated for the human salivary phosphoproteins (Bennick *et al.*, 1981). For ovalbumin we have observed upfield shifts for both SerP residues after addition of $MgCl_2$ (see Fig. 1a and 1b). At saturating concentrations of metal ion the resonance for SerP66 had shifted upfield by 0.25 ppm and the resonance for SerP344 had shifted upfield by 0.32 ppm. From inverse plots of the change in chemical shifts versus the concentration of $MgCl_2$ we deduced that the Kd's were approximately 20 mM and 10 mM for residues 66 and 344 respectively.

Proton-phosphorus coupling constant. Figure 4 shows the difference for a proton coupled and decoupled spectrum of ovalbumin. Clearly the signal to noise ratio in the decoupled spectrum is far better considering that fewer acquisitions were necessary to obtain the spectra. The triplet pattern arises from a coupling to both protons of the α methylene group with a coupling constant *JPH* of 6.5 Hz. Although the coupling can be most easily observed for the resonance of residue SerP344 it is also present for residue SerP68. The same value for the coupling constants were

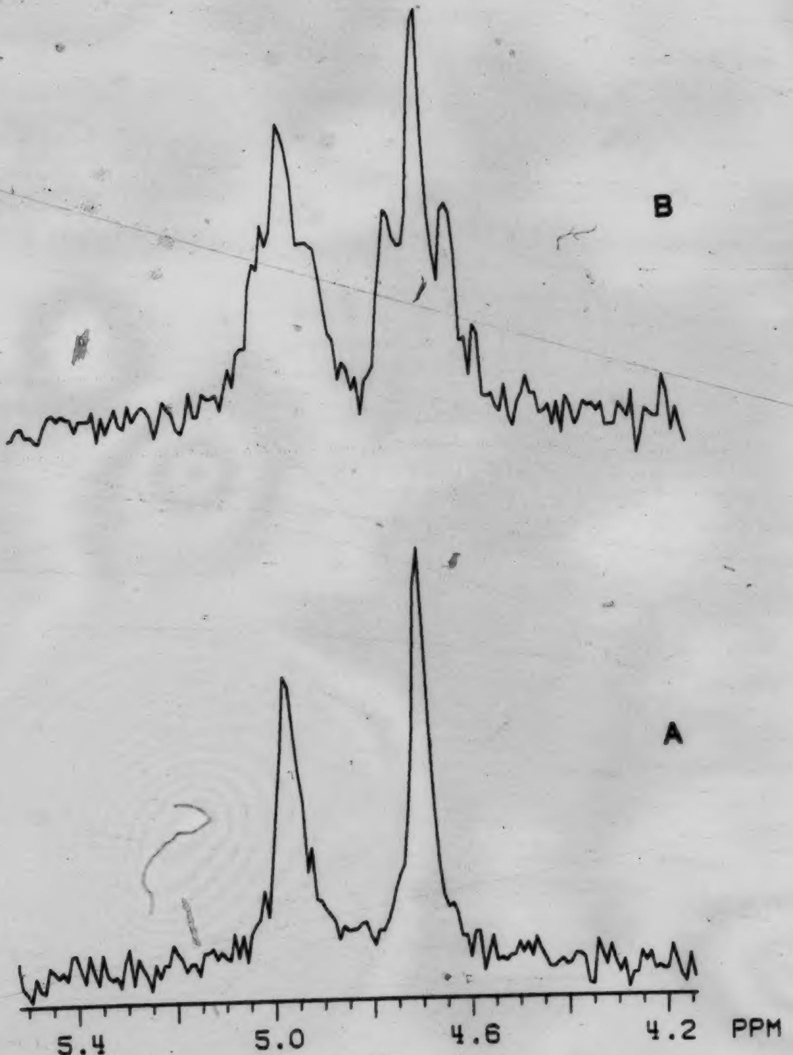

Fig. 9.4 A comparison of the proton-decoupled (A, 1000 scans) and proton-coupled (B, 3000 scans) ^{31}P-NMR spectra (109.3 MHz) of hen egg-white ovalbumin. Additional linebroadening due to the computer digital filtering was 0.25 Hz for both spectra.

obtained from proton coupled spectra obtained at 81 and 162 MHz and from a sample of a phosphoserine standard. Figure 5 shows that the linewidth of residue SerP68 has increased so much by changing to a spectrometer operating at 162 MHz to the extent that the proton splitting can no longer be observed. Such behaviour is diagnostic for major contribution of chemical shift anisotropy to the relaxation of the phosphorus nucleus. This will be considered in detail in the next section.

Frequency dependent measurements. A comparison of Fig. 4 and 5 clearly indicates that the linewidth for both resonances has increased at higher magnetic field strength. A similar field dependence has been observed for other phosphate residues covalently linked to proteins and originates in a CSA contribution to the relaxation of the phosphorus nucleus (see Chapter 4). Figure 6 shows the results we obtained at five different field strengths. It may be seen that the linewidth increases with the increase in the phosphorus resonance frequency. The intercepts on the y-axis show the contribution by field independent proton-phosphorus dipole-dipole coupling. Using the formulae outlined in Chapter 4, and substituting an anisotropy term of 110 ppm (Table 4.2) we have been able to calculate the correlation times from the CSA contribution to the linewidth for the phosphoseryl residues 66 and 344. We have deduced a

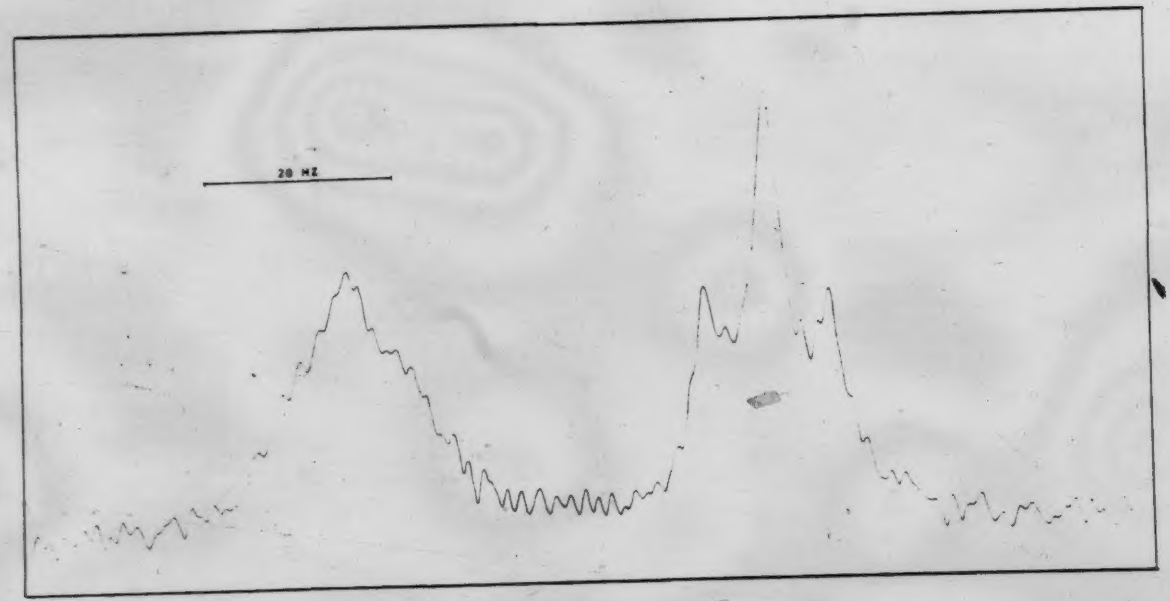

Fig. 9.5 Proton-coupled 162 MHz ^{31}P-NMR spectra of 1 mM hen egg-white ovalbumin at pH 8.3. Conditions were: 15,000 acquisitions and no additional line-broadening.

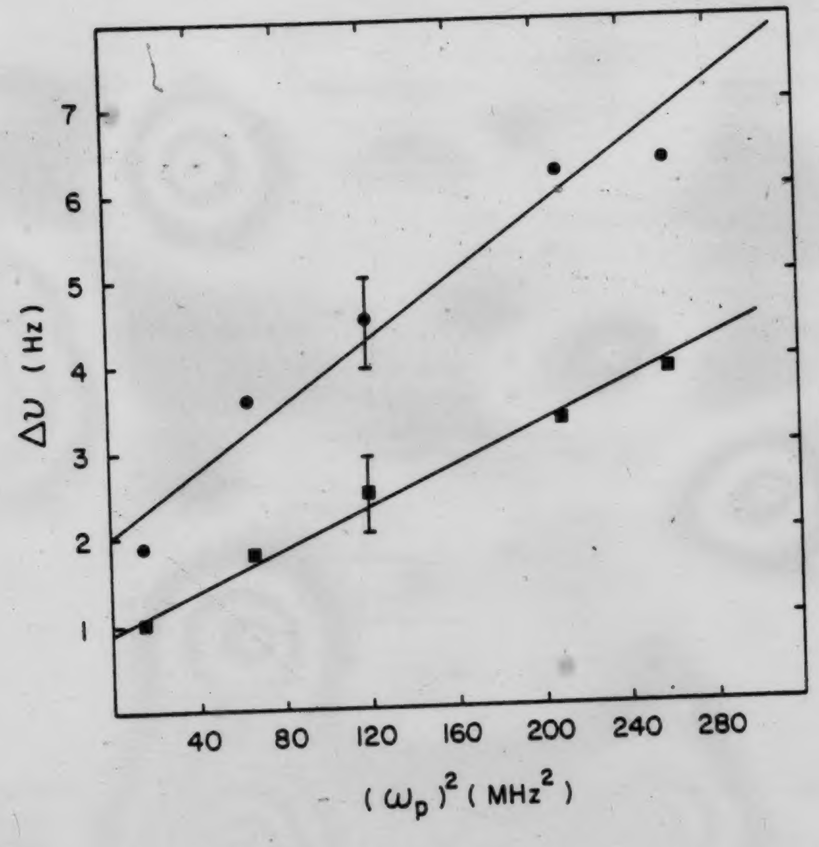

Fig. 9.6 Frequency dependence of the linewidths measured for SerP68 (●) and SerP344 (■). Linewidth were measured at half height and instrumental line broadening was subtracted. The error bars for the points measured at 109.3 MHz indicate the minimum and maximum values measured at this frequency for a wide variety of samples.

correlation time of 15 nsec and 8 nsec respectively.[3] We also attempted to determine the nuclear overhauser enhancement at 109.3 and 145.7 MHz. Its contribution was negligible at both frequencies.

Discussion

The overall rotational correlation time for a protein the size of ovalbumin is 25 nsecs at the temperature of our experiments (28°C) (Yguerabide *et al.*, 1970). The rotational correlation time we have determined for the two phosphoryl groups were 8 and 15 nsecs respectively. Especially residue 344 must therefore have considerable flexibility, but also SerP68 is not fully restricted in its mobility on the protein surface. Flexibility in the sequence of ovalbumin around residue SerP344 had been previously implied by proteolysis studies with subtilisin. Under appropriate conditions ovalbumin will release only a heptapeptide corresponding to the residues 346-352 (Nisbet *et al.*, 1981). Thus this area must be particularly exposed, and our data showing the flexibility of SerP344 agree nicely with this concept. It is of interest that we have found for these mobile residues that there is a contribution of chemical shift anisotropy to the relaxation of the phosphorus nucleus. The relaxation of phosphorus nuclei in small compounds is still a debated subject and it is not clear whether CSA may play a role (McCain & Markley, 1980; Nanda *et al.*, 1980).

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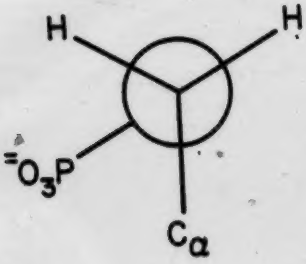
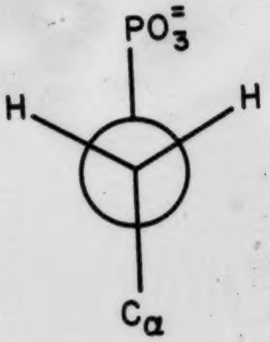
[3] Note that no corrections were made for the natural linewidths. With the approach used here this contribution would be considered part of the proton-phosphorus dipole-dipole relaxation.

SerP344 bears closer resemblance in terms of chemical shift to phosphoserine standards than does SerP68. However the resemblance is still not perfect (4.75 ppm for the SerP344 residue versus 4.50 ppm for the standard) and we have not been able to find a satisfactory explanation for this phenomenon. The pH titrations (Fig. 3) did not reveal the presence of proximal charged groups, nor did unfolding of the protein in urea cause a change in the chemical shift. The main effect of the addition of urea was that residue 344 showed more but not complete resemblance to residue 66 as indicated by a change in linewidth and chemical shift (see Fig. 4).

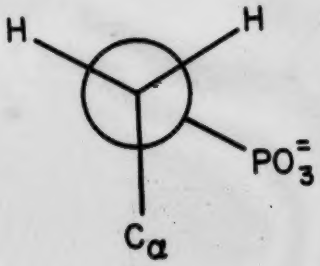
Also of interest was the coupling constant measured for the proton phosphorus splittings for both residues of the protein. The ^{1}H-C-O-^{31}P fragment is known to have an angular dependence of the ^{31}P-^{1}H spin spin coupling which resembles the well known Karplus relationship for ^{1}H-^{1}H couplings (Hall & Malcolm, 1972; Sarma *et al.*, 1973; Blackburn *et al.*, 1973; Evans & Sarma, 1974). Three different rotomers are possible as indicated by the following structures.



I



II



III

Structure II is reported to have a *JPH* of 3 Hz whereas the structures I and III each have a coupling constant of 25 Hz (Sarma *et al.*, 1973; Blackburn *et al.*, 1973). The value of 6.6 Hz we measured for the protein bound residues is the same as that measured for a phosphoserine standard. This value indicates that the rotomer II predominates and that rotation of the phosphoryl moiety is allowed. Hence, this gives further support to our idea that the two phosphoserines of ovalbumin are quite mobile. In contrast, the active site serine phosphate residue of apo-alkaline phosphatase had a *JPH* of 13 Hz indicating that rotomers I or III are present (Chlebowski *et al.*, 1976).

The pH titratability, combined with the data from our phosphatase digestions indicate that both residues are at the surface of the protein. Alkaline phosphatase digestions have been attempted by others for a variety of phosphoproteins. The results indicated that the majority of such sites can be hydrolysed by the *E. coli* enzyme (Swarup *et al.*, 1981; Bennick *et al.*, 1981). Thus the majority of these phosphate residues are probably located on the surface of proteins. We have shown, by performing phosphatase digestions at different pH values, that ovalbumin may undergo a pH dependent conformational change in which SerP68 becomes shielded at higher pH but is more exposed at lower pH. Further support for such a conformational change comes from pH-dependent shielding and deshielding of aromatic residues as implied by photo laser CIDNP experiments (H.J. Vogel, unpublished observations). Since both phosphates are exposed at the surface it is not surprising that they can bind metal ions. The Kd's of 20 and 10 mM respectively observed for Mg^{2+} binding at pH 8.3 do not support a role of the two phosphoryl moieties in a special metal binding site as in human salivary phosproteins. (Bennick *et al.*, 1981). Thus these data indicate no role of ovalbumin involving the storage of metal ions.

Amino acid modification studies of the positively and negatively charged amino acids have indicated that the large majority of such residues are shielded from the solvent (Taborsky, 1974). Thus the two exposed phosphate residues must make a major contribution to the surface charge of the protein. This may explain why ovalbumin can be so readily separated in its bis-, mono-, and dephosphorylated forms by electrophoresis under nondenaturing conditions (Linderstrøm-Lang and Ottesen, 1949; Perlmann, 1955). It is not clear at present whether this same property can explain the heterogeneity which is sometimes observed upon SDS polyacrylamide gel electrophoresis of otherwise apparently homogeneous preparations of ovalbumin (Atkinson *et al.*, 1981).

Recently it was reported that parts of the ovalbumin sequence were homologous to those of the protease inhibitors antithrombin III and α_1-proteinase (Hunt & Dayhoff, 1980). Thus it was speculated that the protein could serve as protease inhibitor, but this was not borne out by subsequent experiments (Long and Williamson, 1980). Here we want to draw attention to another homology. The site around serine phosphate 68 bears a close resemblance to active site peptides isolated from serine proteases, alkaline phosphatases, esterases and acyl-carrier protein (see Table 2). All of these have a glycine followed by an acidic residue, then the "active" serine residue followed by a hydrophobic amino acid. No resemblance exists with phosphoglucomutase and the subtilisin-type protease active sites. Thus one might speculate that the site around serine 68 in ovalbumin is a remnant of a possible hydrolytic active site. Since the protein is homologous to protease inhibitors as well, it could be further speculated that ovalbumin may resemble a protease inhibitor complex.[3] It is interesting in this

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[3] Other considerations make this possibility quite unlikely. All serine proteases contain a second active site peptide which is usually Ala-Ala-His-Cys (James *et al.*, 1978). Inspection of the sequences surrounding the 7 ovalbumin His residues reveals only one peptide with some homology Ala-Ala-His-Ala. A change from an Ala to a Cys residue requires minimally two base pair changes. Moreover the alignment of this peptide (His331) with respect to that of the Ser peptide (Ser344) is different from that in serine proteases.

Table 9.2

Comparison of the amino acid sequence of the ovalbumin serine phosphate peptides to active site peptides of hydrolytic enzymes

Ovalbumin SerP344	Val-Gly-Ser-Ala
SerP68	Gly-Asp-Ser-Ile
Alkaline Phosphatases	
- *E. coli*	Thr-Asp-Ser-Ala
- Calf Intest.	-Asp-Ser-Ala
- *S. marcescens*	Thr-Asp-Ser-Ala
Serine Proteases	
- trypsinogen	Gly-Asp-Ser-Gly
- chymotrypsinogen	Gly-Asp-Ser-Gly
- elastase	Gly-Asp-Ser-Gly
- thrombin	Gly-Asp-Ser-Gly
- α lytic protease	Gly-Asp-Ser-Gly
- *Strep. griseus* protease A	Gly-Asp-Ser-Gly
- *Strep. griseus* protease B	Gly-Asp-Ser-Gly
- *Strep. griseus* trypsin	Gly-Asp-Ser-Gly
- subtilisin	Gly-Thr-Ser-Met
- aspergillopeptidase	Gly-Thr-Ser-Met
Esterases	
- carboxylesterase	Gly-Glu-Ser-Ala
- acetylcholinesterase	-Glu-Ser-Ala
- pseudocholinesterase	Gly-Glu-Ser-Ala
Acyl carrier protein *E. coli*	-Asp-Ser-Leu
Phosphoglucomutase	Thr-Ala-Ser-His

All data obtained from James *et al.* (1978) and the CRC Handbook of Biochemistry.

respect that it has been shown that the location of an acid residue preceding the SerP is highly conserved. The peptide sequences determined for ovalbumins purified from eight different sources all showed the following sequence: Gly-Asp or Glu-SerP-Ile or Val or Met (Henderson *et al.*, 1981). Moreover the authors comment that the amino acid sequences around the phosphoserines show a slower rate of mutation than the sequences of the remainder of the molecule.

A second point with respect to the sequence is of interest as well. Henderson *et al.* (1981) showed that in all 16 phosphopeptides isolated from the 8 different ovalbumins the residue in position n + 2 is always glutamic acid. Moreover all Ser-X-Glu or Ser-X-SerP sequences in α and β caseins are always phosphorylated (Mercier *et al.*, 1971; Ribadeau Dumas *et al.*, 1972). Thus this negatively charged residue in position n + 2 may be a recognition point for a protein kinase. This view is especially supported by studies on genetic variants of casein. In one of these the Glu37 residue is replaced by a Lys. Significantly the Ser35 is not phosphorylated in this variant, while it is phosphorylated when a Glu is in place (Grosclaude *et al.*, 1972). It has been outlined that generally positively charged entities placed in position n+2 or n-2 are involved in the recognition by protein kinases (Williams, 1976). However the data presented above are in support of a separate protein kinase specifically recognizing negative charges at position n+2.

Furthermore, phosphorylation sites within troponin I (Williams, 1976) and human fibrinogen (Taborsky, 1974) contain an acid residue at position n+2 from a phosphorylated serine. Extending this argument to phosvitins and protamines suggests that a SerP residue could sometimes support phosphorylation by such a kinase, but these two molecules probably require another kinase for phosphorylation. Their common feature is a positively charged residue directly succeeding a SerP or a stretch of SerP residues (Taborsky, 1974; Williams, 1976). It should be noted that ovalbumin contains three additional Ser-X-Glu peptides in its overall sequence that are not phosphorylated. It is possible that these sites are located on the inside of the molecule and cannot be reached by the protein kinase. Studies on these Ser-X-Glu specific kinases from either mammary gland or chicken oviduct might aid in understanding the biological function of the structural phosphorylations of caseins and ovalbumins.

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CHAPTER 10

^{31}P-NMR STUDIES OF *E. coli* CONJUGATIVE PILI

Summary

Conjugative pili isolated from *E. coli* strains harbouring the derepressed EDP208 and ColB2 derivatives of the F-plasmid were studied by ^{31}Phosphorus nuclear magnetic resonance spectroscopy. Studies in dissociative buffers (1% SDS) revealed a resonance with a chemical shift position and pH titration characteristics indicative of a phosphodiester. Mn^{2+} induced, EDTA reversible, broadening of the resonance observed for this protein associated compound suggested that the residue was accessible to solvent. Acetone treatment of the protein resulted in the liberation of the phosphodiester and subsequent identification by thin-layer chromatography as a mixture of phospholipids. Analysis of the observed linewidths for these pilin-associated phospholipids indicated that they were tightly bound and had no residual mobility with respect to the protein surface. Also, the pilin monomers seem to aggregate in a concentration dependent manner. Thus 1% SDS appears to be ineffective in dissociating the protein into its monomers at higher protein concentrations. These findings are discussed with respect to recent observations by ^{31}P-NMR of phosphodiesters associated or possibly covalently attached to other proteins.

Introduction

Pili are a diverse group of non flagellar filamentous appendages found on many strains of bacteria. Two groups are usually distinguished. They appear to be unrelated [1]. The first group is that of the "common pili", these are of general occurrence and are thought to play a role in adhesiveness and sensitivity to phage infection etc. The other group is that of the "conjugative pili". These are encoded for by small self transmissible plasmids and are the topic of this study. They are only found on the surface of male cells and their role lies possibly in initiating cell-to-cell contact during bacterial conjugation [2,3]. They also act as the attachment site in the infectious process of several male-specific bacteriophages [1]. These pili are composed of single subunits, called pilin, (MW = 12,000), arranged in a helical fashion to give thin tubular filaments 8 mm in diameter and approximately 1 uM in length [4]. A pool of such subunits is located in the inner membrane [5], although very little is known yet about the process of assembly and disassembly.

EDP208 as well as ColB2 conjugative pili are encoded by derepressed derivatives of the naturally occurring F-plasmid. *E. coli* strains harbouring such plasmids generally produce greater quantities of conjugative pili than normal strains that produce only one F-pilus per cell. Thus such strains allow purification of large amounts of pili material [1,6]. It has been shown by comparison of amino acid analysis, peptide fingerprints, phage sensitivities and X-ray diffraction patterns that both kinds of pili resemble the F-pili [1,6]. All these conjugative pili were reported to contain covalently bound phosphorus, albeit with different stoichiometry [1,2]. Here we report on ^{31}P-NMR studies primarily aimed at resolving the nature of the linkage involved.

Experimental Procedures

The pili used for these studies were purified from the multipiliated strains EDP208 and ColB2 and contained approximately 3 moles of phosphate per mole of pilin [1,6]. They were a gift from Drs. G.D. Armstrong and W. Paranchych. Due to the poor solubility properties of ColB2, the majority of the experiments were performed on pili obtained from EDP208. All samples were dissolved in a buffer containing: 100 mM Tris, 1 mM EDTA, 1% SDS, 25% D_2O (Biorad) at pH 8.3 unless otherwise indicated in the text. All experiments not dealing with NMR were performed by Dr. G. Armstrong and are described elsewhere [6]. ^{31}P-NMR spectra were obtained at a frequency of 109.3 MHz on a Bruker HXS-270 spectrometer, interfaced with a Nicolet-1080 computer and operating in the Fourier transform mode. The spectrometer was locked on the deuterium resonance of the 25% D_2O included in all samples. The chemical shifts are referenced to an external standard of 85% phosphoric acid, downfield shifts are given a positive sign. All spectra were recorded at 28 ± 1°C in 1.5 ml samples placed in 10 mM NMR tubes (Wilmad). A typical instrument setting was: pulse angle 60°, acquisition time 0.4 sec., relaxation delay 1.6 sec., sweep width 5,000 Hz. When proton-decoupling was used (see text), the decoupler was on only during acquisition (4 watts), irradiating an area corresponding approximately to 3.5-6.5 ppm in a ^{1}H NMR spectrum.

Results

Our original attempts to observe a phosphate resonance for intact EDP208 pili were unsuccessful. Since the overall correlation time for intact pili (MW >> 10^{7}) will be very long this may result in an ^{31}P-NMR resonance that is too broad to be observable, if the residue is tightly held by the protein. All subsequent experiments were therefore done with a buffer containing 1% SDS which is reported to dissociate the pilus into its pilin subunits [2]. Figure 1a shows the spectrum obtained for a 12 mg/ml solution. A major peak is centered around 1 ppm and a minor peak around 0.6 ppm. Figure 1b shows the effect of ^{1}H decoupling. Comparison of Fig. 1a and 1b shows that the lines are narrower with the decoupler turned on. Such behaviour is usually observed for phosphomono- and -diesters and not for other naturally occurring linkages (H.J. Vogel, unpublished observations). These two esters can be distinguished in an ^{31}P-NMR spectrum on the basis of the chemical shift and pH

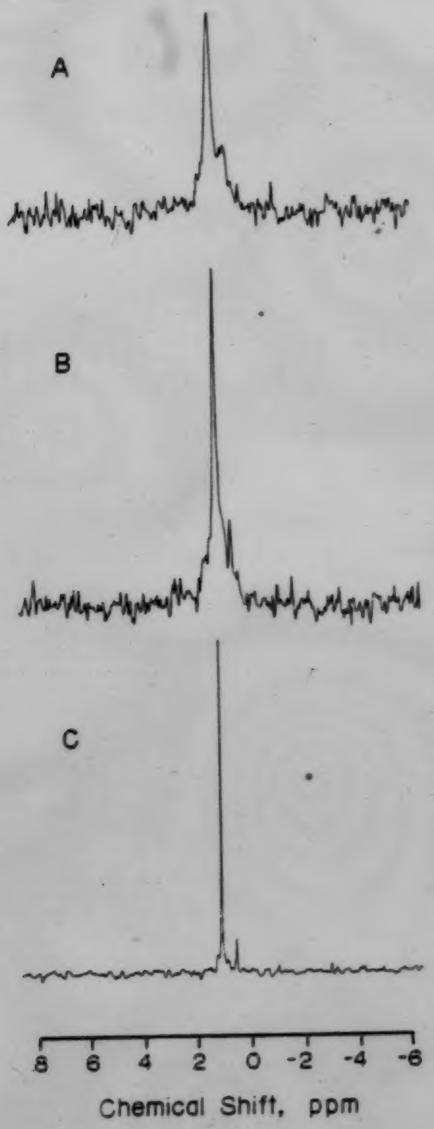

Fig. 10.1 ^{31}P-NMR spectra of 12 mg/ml EDP208 pili. Each spectrum represents 300 scans. For (a) and (b) extra line broadening of 2.5 Hz was used, for (c) 1 Hz. (a) normal ^{31}P-NMR spectrum; (b) proton-decoupled spectrum; (c) protondecoupled spectrum of the resuspended acetone supernatant. Other conditions were as indicated in experimental procedures.

titration behaviour. The chemical shift of 1.0 ppm is compatible with either a phosphodiester or a monoanionic phosphomonoester. When, for a similar sample, the pH was varied between 10 and 6.2 no changes in chemical shift were observed (at lower pH's the protein precipitated). A phosphomonoester would have shifted under these conditions, thus this behaviour is indicative of a phosphodiester [7,8]. Another possible explanation is that the residue might be inaccessible to solvent and does not shift for that reason. Such behaviour has been observed for many protein bound residues [9]. To investigate this further we added a small amount of $MnCl_2$ to another sample (without EDTA in the buffer). This caused broadening of the line beyond detection, the addition of a sufficient amount of EDTA reversed this effect. This observation makes it less likely, but does not exclude, that the phosphorus is internally bound and does not shift for that reason [10]. In a next experiment the sample of Fig. 1b was treated with acetone. This caused the protein to precipitate and it could be removed by centrifugation. Phosphate analysis at this point indicated that the phosphate was quantitatively transferred into the acetone supernatant. This suggested that the phosphate containing material was not covalently linked to the protein, or that perhaps a phosphoryl bond was broken due to the treatment. After evaporation of the acetone with a stream of nitrogen, the remaining material was resuspended in the original buffer. The proton decoupled spectrum observed for this material is shown in Fig. 1c. Noteworthy is that the resonance is very narrow, equivalent to that of small molecules in solution. Moreover it has not shifted indicating that no bonds have been broken, so it appears that the material was not covalently linked to the pilin subunits. When titrated between pH 12.8 and pH 3.0, the resonance of Fig. 1c did not shift. Prolonged exposure of the material to base resulted in the appearance of new peaks in the spectrum with chemical shift position and pH dependent changes in the chemical shift corresponding to those of a phosphomonoester and inorganic phosphate (data not shown). This base sensitivity [11] confirms that the contaminant contains a phosphodiester linkage.

Results obtained with ColB2 pili were essentially the same. One major difference was noted, however. This is illustrated in Fig. 2. Since the two spectra shown are taken from samples with the same protein concentration and all other conditions equal, they can be directly compared. From the difference in linewidth (resulting in a change in the signal to noise ratio) it appears that the phosphate bound to ColB2 has a higher mobility than those bound to EDP208. Another interesting result becomes apparent from a comparison of Fig. 1b with 2a. Dilution of the EDP208 sample from 12 mg/ml to 2 mg/ml has resulted in a decrease in linewidth from 17 Hz to 10 Hz respectively. For a sample of 25 mg/ml EDP208 a linewidth of 45 Hz was observed. Thus the linewidth

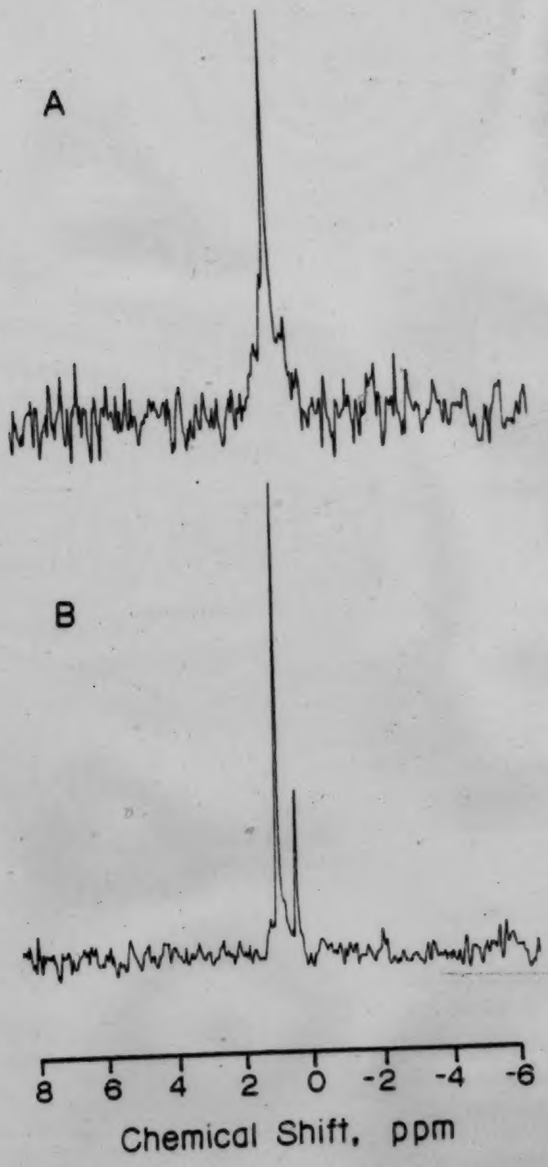

Fig. 10.2 A comparison of the proton decoupled ^{31}P-NMR spectra of EDP208 (a) and ColB2 (b). Both samples contained 2 mg/ml of protein, and approximately 3 mol of phosphate per mole of pilin. Each spectrum has an added linebroadening of 2.5 Hz, 2,500 scans were taken for both (a) and (b).

observed for this phosphodiester moiety increases drastically with the protein concentration.

Discussion

All the data we have presented on proton decoupling, the pH independence of the chemical shift and the base sensitivity are consistent with our conclusion that the resonance observed in the ^{31}P-NMR spectra of conjugative pili originates from a phosphodiester bond. Since acetone treatment quantitatively removes this ^{31}P resonance from the protein, apparently without breaking any bonds, we concluded that this phosphodiester is not covalently linked to the protein in contrast to earlier suggestions [1,2]. Our ^{31}P-NMR data do not distinguish between different biological molecules that contain such linkages, like RNA, DNA or phospholipids [7,8]. The absence of any 260 mn UV absorbance rules out these first two possibilities. Subsequent thin layer chromatography experiments confirmed that the material in the acetone supernatant comigrated with the phospholipids phosphatidylglycerol (53%), phosphatidylethanolamine (30%) and phosphatidylserine (13%) [6]. It is noteworthy that this differs significantly from the composition of membranes of the *E. coli* strain from which these pili were isolated (90% phosphatidylethanolamine, and only 10% phosphatidylglycerol) [6]. This observation accentuates the question whether the attachment of phospholipids plays a biologically relevant role in the conjugation process or if it is an artifact that is introduced by the purification procedure.

Since treatment with 1% SDS or acid precipitation did not result in release of the phospholipid, we conclude that the pili have a strong affinity for such compounds, only treatments with acetone or chloroform/methanol mixtures resulted in extraction. Phosphodiester resonances were recently identified in the ^{31}P-NMR spectra of *Aspergillus niger* glucose oxidase [10] and *Azotobacter* flavodoxin [12]. These findings were taken as evidence for the existence of a phosphobridge linking two amino acid residues of one polypeptide. Since in both cases no extractions with organic solvents were tried we feel that the possibility of contaminating phospholipid is not adequately ruled out, as indicated in the present observations. Moreover, X-ray crystallographic studies and amino acid sequencing studies of flavodoxins purified from related organisms [13] have not provided evidence for the existence of such a linkage. Thus the intriguing possibility that phosphobridges may provide links between polypeptides similar to disulfide links definitely awaits further confirmation by isolation and identification of such a phospho-diamino acid from pronase digests of these proteins.

Our measurements allow us to draw some conclusions about the mobility of the bound phospholipid. At a concentration of 2 mg/ml, a linewidth of 10 Hz was observed (Fig. 2a). At the spectrometer frequency used (109.3 MHz) the linewidth will be mainly determined by Chemical Shift Anisotropy (CSA) [14,15]. Indeed an estimation of the proton-phosphorus dipole-dipole relaxation, using the formula's of chapter 4, yields a linewidth of only about 1 Hz. Thus the residual 9 Hz is the CSA contribution to the linewidth. From this we calculated, substituting an anisotropy term of 170 ppm which is a normal value for phosphodiesters [14,15], that the rotational correlation time is 24 nsecs. A normal rotational correlation time determined for a protein of this size at this temperature but in the absence of SDS is 10 nsecs [16]. Thus the phospholipids appear to be rigidly held by the pilin subunits and have no residual mobility with respect to the protein surface. This explains why we failed to detect the spectrum of intact pili, the resonance simply becomes too broad to be observable. It should be emphasized here that binding of SDS to the protein can drastically alter its molecular weight and correlation time. Thus our calculated rotational correlation times cannot be directly compared to those measured for proteins in the absence of SDS. Aggregation may occur as indicated by the large increase in linewidth observed at higher protein concentrations. This last observation suggests that 1% SDS does not fully dissociate the EDP208 pili into its monomers as was previously suggested [2]. It should be mentioned here however that SDS-polyacrylamide gel electrophoresis studies with protein concentrations up to 1 mg/ml show that the majority of the protein migrates as a monomer. From Fig. 2a and 2b we concluded that the phosphate group of ColB2 has more mobility than that of EDP208. Organic solvents, however, were less effective in extracting the phospholipids [6]. Thus the binding of these to ColB2 is as tight or even tighter than to the EDP208 pilin subunits. The narrower linewidth observed then indicates that the ColB2 subunits either do not aggregate under these conditions, or that the phosphodiester-portion of the phospholipid has some mobility relative to the protein surface. It is interesting that these two functionally similar proteins differ in such a property.

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CHAPTER 11

THE STRUCTURE OF HEN PHOSVITIN: A ^{31}P-NMR, ^{1}H-NMR AND PHOTO-LASER CIDNP ^{1}H-NMR STUDY

Summary

^{1}H-NMR studies of hen egg yolk phosvitin (MW = 35,000) have confirmed earlier suggestions that the protein has a very open structure and is not compactly folded. More than half of a total 220 residues in this unusual protein are phosphoserine residues. ^{31}P-NMR pH titration studies have shown that all of these are in the dianionic form at physiological pH and that they are exposed to solvent and can be protonated and deprotonated. The pKa's determined for several groups of resonances that could be resolved in the ^{31}P-NMR spectra were all close to those observed for phosphoserine standards. The low Hill coefficient ($n = 0.70$) calculated for these titration curves indicate interactions between the negatively charged phosphoryl moieties. Small inflections in the titration curves observed at pH 10.5 suggest that arginine and lysine residues interact with the phosphoryl moieties.

Subsequent photolaser CIDNP ^{1}H-NMR experiments suggest that the single tyrosine and tryptophan residues were both exposed to solvent. Surprisingly, all eleven histidine residues appear shielded from the solvent since only very

small CIDNP effects were observed for these residues. Further experiments indicated that the presence of tryptophan or tyrosine in mixtures of aromatic amino acids decreases the CIDNP intensities observed for histidine residues. Thus this effect may have led to the incorrect conclusion that all histidine residues are shielded in phosvitin. However, subsequent experiments comparing the CIDNP spectra for phosvitin with those for mixtures of aromatic amino acids resembling the composition of this protein demonstrated that the histidine residues are more shielded in the intact protein. Since proton abstraction is necessary for CIDNP effects of histidine residues, these observations suggest that the histidine residues may be protonated and in salt linkage to the phosphoryl moieties. This concept was further supported by the results of ^{1}H-NMR titration studies that indicated that all eleven histidines have an unusually high pKa = 7.45 and a low Hill coefficient for titration of n = 0.80. Thus in the absence of metal ions the structure of phosvitin is viewed to consist of an extensive network of charge and hydrogen bonded interactions that are in rapid exchange between all phosphoryl moieties. Our CIDNP and ^{1}H-NMR titration studies have allowed the assignment of various resonances in the aromatic region (6-9 ppm) of the ^{1}H-NMR spectrum.

Introduction

Both major egg yolk proteins phosvitin and lipovitellin are derived from a common precursor called vitellogenin. This protein is synthesized in the livers of all egg laying animals. Its synthesis is under hormonal control; administration of estrogen has been reported to increase the rate of transcription (Tata, 1976, Ryffel, 1978). Vitellogenin undergoes extensive posttranslational modifications: phosphorylation, glycosylation and attachment of lipid moieties. After synthesis and processing in the liver vitellogenin is transported through the blood to the ovary where it is cleaved to yield the egg yolk proteins before they are deposited in the yolk (Tata, 1976; Ryffel, 1978). Each vitellogenin molecule gives rise to two phosvitin molecules (Deeley *et al.*, 1975). Highly purified preparations of phosvitin all show some heterogeneity (Taborsky, 1974). This property appears to be mainly related to the existence of different vitellogenin genes (Wiley and Wallace, 1981). The extent of the posttranslational modifications appears to be constant at least in preparations of hen phosvitin (Taborsky, 1974). Phosvitin (MW = 35,000) has a very unusual amino acid composition (see Table 1). More than half of the residues are phosphoserines, accompanied by a fairly large number of acidic amino acids. The basic amino acids that are present are insufficient to balance all the negative charges and thus the protein is a polyelectrolyte.

Table 11.1

Amino acid composition of hen phosvitin

Residue	Number of residues per mole of protein
Gly	7
Ala	7
Val	3
Leu	3
Ile	2
Pro	3
Phe	2
Tyr	1
Trp	1
Ser	120
Thr	5
Cys	0
Met	1
Asx	13
Glx	13
His	11
Lys	17
Arg	11
(P)	(112)
Total	220

The data are the average of the 6 independent determinations described by Taborsky (1974).

Parts of the protein have been sequenced (Belitz, 1965). These studies have led to the surprising discovery that blocks of usually 6 but sometimes up to 8 SerP residues may occur in one stretch. Such a stretch is usually terminated by a basic residue. Moreover these blocks of SerP residues are evenly spread throughout the primary structure. This amino acid sequence provides for a protein with a very atypical structure. Its partial specific volume is only 0.54 ml/gr (a "typical" protein value is 0.73 ml/gr). Hydrodynamic measurements suggested an elongated shape whereas optical rotatory dispersion and circular dichroism studies indicated that no extensive α-helix or β pleated sheet structures are present at pH values where the phosphate residues are expected to be doubly negatively charged (for review see Taborsky, 1974).

The biological role of phosvitin is not well understood. It has been suggested that it may serve as an iron (Fe^{3+}) carrier in blood (as part of vitellogenin, Greengard *et al.*, 1964; Ali *et al.*, 1968) as well as in eggs (Taborsky, 1980). It has been shown that the protein can tightly bind Fe^{3+} (Hegenauer *et al.*, 1978) at a ratio of Fe:P of 1:2 suggesting that diSerP units are effective in the binding. SerP itself has no affinity for Fe^{3+} ions and diSerP units turned out to be much less effective in the sequestering of Fe^{3+} than phosvitin itself (Donella *et al.*, 1976; Hegenauer, 1978). Thus other features of the protein must aid in the stabilisation of the binding of this metal ion. This Fe^{3+} binding is reported to occur with different affinities and occurs intramolecularly (Taborsky, 1980). In contrast, binding of Mg^{2+} and Ca^{2+} occurs in a one to one ratio with respect to phosphorus and appears to result in the formation of intramolecular crosslinks (Grizutti and Perlmann, 1973, 1975). Various spectroscopic studies have suggested that the protein would be highly ordered in the presence of bound Fe^{3+} (Webb *et al.*, 1973; Gray, 1975). However recent CD measurements have not shown extensive changes in the polypeptide backbone conformation upon Fe^{3+} complexation (Taborsky, 1980).

Here we report on ^{1}H-NMR, ^{31}P-NMR and photo-laser-CIDNP ^{1}H-NMR studies of hen phosvitin. Our studies have confirmed that the protein has an unfolded structure which is ordered somewhat by an extensive network of rapidly exchanging charge and H-bond interactions. Earlier ^{31}P-NMR studies had indicated that the majority of the phosphate residues are present in a SerP linkage and that the observed resonances shift with changes in pH (Ho *et al.*, 1969).

Experimental procedures

Phosvitin was obtained from Sigma Chemical Company and was used without further purification. Protein for ^{1}H-NMR and photo-laser-CIDNP experiments was usually lyophilised once from D_2O to reduce the HDO resonance in the ^{1}H-NMR and photo-laser-CIDNP experiments. The samples were then dissolved in a buffer containing 50 mM KH_2PO_4, 0.05 mM EDTA, pH 7.0, 99.8% D_2O (Biorad). The pH (meter reading) was adjusted by adding small aliquots of 1 N NaOD and DCl solutions made up in 99.8% D_2O. Samples were centrifuged at 2000 rpm for 15 min to remove any insoluble material. The pH was measured on a Radiometer PHM62 standard pH meter equipped with a glass electrode. Samples for ^{31}P-NMR were directly dissolved in a buffer containing 50 mM Tris/HCl, 50 mM maleic acid, 1 mM EDTA, 35% D_2O at pH 8.3.

^{31}P-NMR spectra (proton-decoupled) were recorded on a Bruker HXS-270 spectrometer at a frequency of 109.3 MHz. Other conditions have been described elsewhere (Vogel and Bridger, 1982). Samples of 1.5 ml were placed in 10 mm round bottom precision tubes (Wilmad) equipped with teflon Vortex plugs. All chemical shifts are referenced to 85% H_3PO_4. Upfield shifts are given a negative sign. ^1H-NMR and photo-laser-CIDNP ^1H-NMR spectra were obtained at the same spectrometer at a frequency of 270 MHz. ^1H-NMR chemical shifts are referenced to DSS, with a positive sign given to downfield shifts. Samples (1 ml) were placed in 10 mm flat bottom precision tubes fitted with Vortex plugs. The pulse angle applied was approximately 90° and the time between individual pulses for the ^1H-NMR experiments was 0.5 sec. All spectra were obtained with homonuclear broadband decoupling to suppress the HDO resonance. Photo-laser-CIDNP experiments were performed essentially as described by Hincke *et al*. (1981) with the following modifications. All samples contained 5 ul of a 50 mM FMN (Sigma Chemical Company) stock solution in 100% D_2O. Alternating light and dark spectra were obtained and stored and added on the disk (Kaptein, 1978, 1982). Two different sequences were used, but in both a laser pulse of 3.0 watts was used (Spectra Physics model 164 argon ion laser operating in the multiline mode). Sequence 1 employed a 1.0 sec laser pulse before the acquisition of the spectrum and a 20 sec delay between individual light pulses, in sequence 2 a 0.8 sec laser pulse was used which was repeated every 16 sec. Results with either sequence were virtually identical. A series of control experiments with mixtures of aromatic amino acids and the proteins ovalbumin and lysozyme[1] had shown that under these conditions close to optimal CIDNP enhancements were obtained while minimizing bleaching of the dye and modification of amino acid by possible photo-oxidation (see also McCord *et al*., 1981). Since these last two effects influence the intensities observed, fresh samples were used for each photo-laser-CIDNP experiment that involved measuring the intensities. All NMR experiments were performed at a temperature of 28 ± 1°C.

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[1] H.J. Vogel and B.D. Sykes, manuscripts in publication.

Results and Discussion

^{1}H-NMR Studies. Figure 1 shows a ^{1}H-NMR spectrum obtained for a sample of phosvitin. It shows that all the resonances in the aliphatic region of the spectrum are remarkably well resolved for a protein of this size (MW = 35,000). The spectrum bears more resemblance to that of a mixture of amino acids than to spectra published for proteins with similar molecular weight (Wüthrich and Wagner, 1978). The sharp resonance at 0.9 ppm represents all the methyl groups in the protein. The resonance at 4.1 ppm is remarkably high in comparison to spectra obtained for other proteins and reflects the SerP methylene group. Since homonuclear decoupling was used to suppress the residual HDO line in this spectrum, the intensities are likely to be effected by cross relaxation phenomena and thus unfortunately cannot be used for direct estimation of the amino acid composition.

Multiplication of the free induction decays with a sinebell results in line narrowing for sharp resonances and removes broad resonances from the spectrum (Gassner *et al.*, 1978). When we applied this technique to phosvitin, all resonances sharpened in the ^{1}H-NMR spectrum. In contrast similar manipulations of free induction decays collected for ovalbumin samples virtually removed all resonances from the spectrum (data not shown); ovalbumin is compactly folded (Taborsky, 1974) and is thus expected to give rise to broad

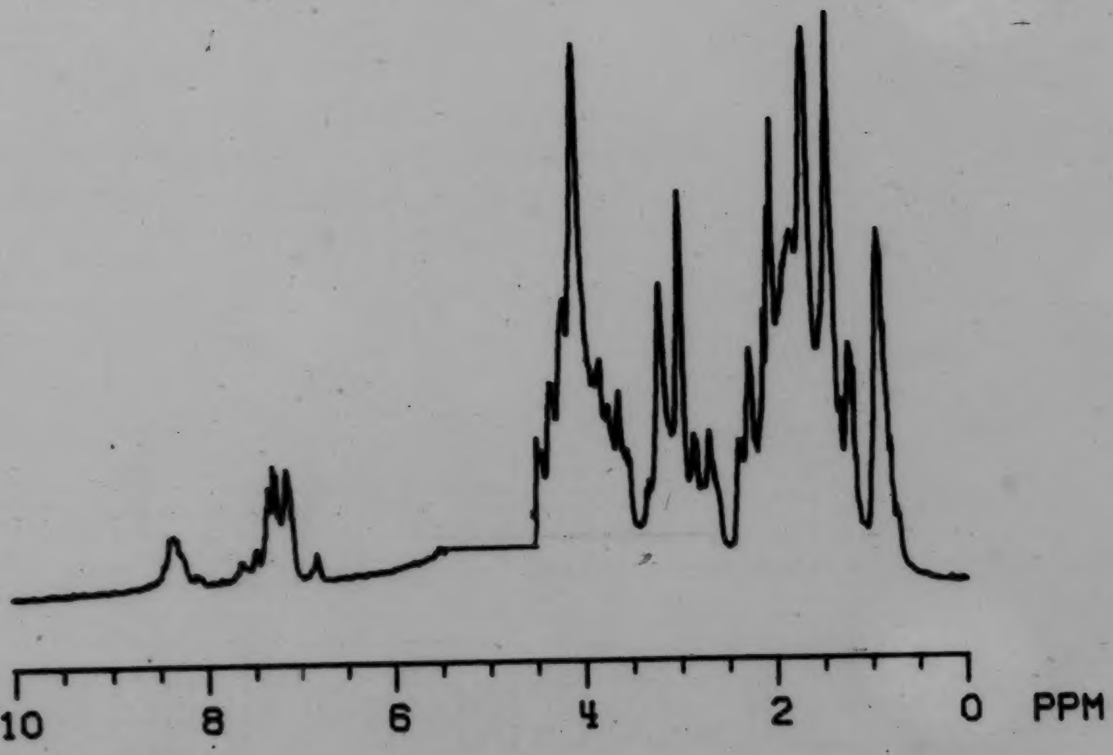

Fig. 11.1 270 MHz ^{1}H-NMR spectrum of hen phosvitin at pH 7.0. 1000 acquisitions were taken of a 0.5 mM phosvitin sample, the delay between individual pulses was 1.4 sec. An additional line broadening of 1 Hz was introduced by the computer digital filtering.

resonances. The above suggests that no broad resonances exist for phosvitin, indicating the protein to be very flexible. This notion found further support in our T_1 inversion recovery measurements performed again on both ovalbumin and phosvitin. In compactly folded proteins cross relaxation (or spin diffusion) will effectively eliminate differences between T_1 values for the different amino acid residues (Kalk and Berendsen, 1976; Sykes *et al.*, 1978). Indeed, all resonances in the compactly folded ovalbumin had identical T_1 values. However, all resonances in the ^{1}H-NMR spectrum of phosvitin had different T_1 values, again suggesting that the protein is unfolded (data not shown). The spectra shown in Fig. 1 and Fig. 2 give no evidence for unexchangable NH residues, even when the protein was dissolved directly prior to the acquisition of the spectrum. This gives additional support to the idea that the protein is unfolded and flexible, since all backbone-NH residues can rapidly exchange with solvent D_2O (Wüthrich and Wagner, 1978).

Assignment of aromatic resonances in the ^{1}H-NMR spectrum. Figure 2 shows the aromatic region of the 270 MHz ^{1}H-NMR spectrum of phosvitin. The resonance centered around 6.79 ppm gives rise to a large emission in a photolaser CIDNP spectrum (see Fig. 6) and thus represents the 3,5 protons of the carboxy-terminal tyrosine residue (Taborsky, 1974). The corresponding tyrosine 2,6 protons appear at

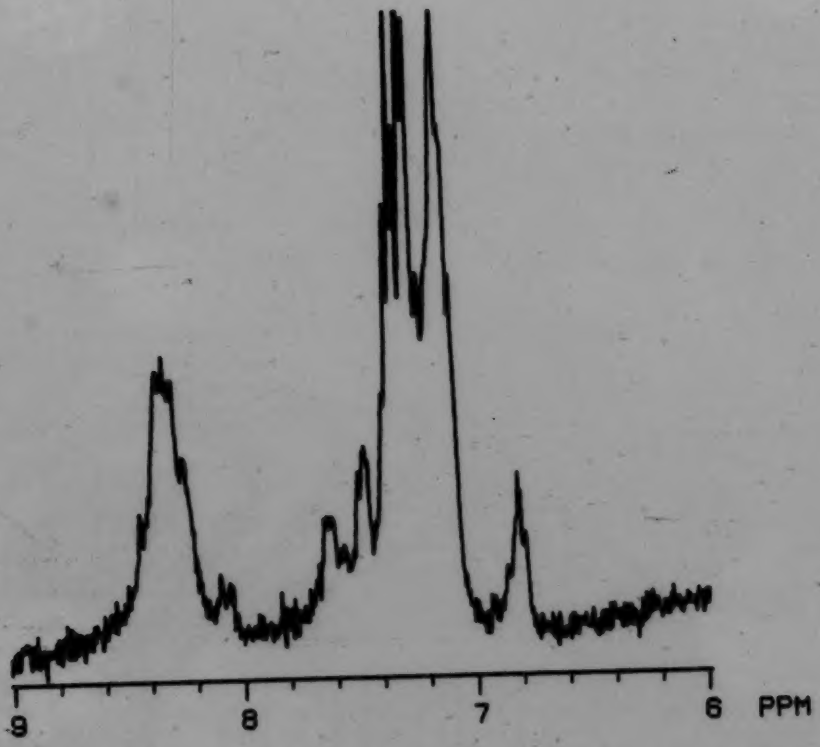

Fig. 11.2 Aromatic region of the 270 MHz ¹H-NMR spectrum of phosvitin (see Fig. 11.1). No additional line broadening.

7.07 ppm. They can be clearly resolved at high or low pH when the His H_4 protons (in Fig. 2 centered around 7.14 ppm) are shifted. The resonances observed for the tyrosine ring protons shift upfield at pH >10, indicating that the tyrosine phenolic residue has a normal pKa. CIDNP spectra as shown in Fig. 6 indicate that the tryptophan H_5 and H_6 resonances appear at 7.22 ppm and the H_4 resonance appears at 7.62 ppm. The resolved resonance observed at 7.48 ppm probably corresponds to the tryptophan H_7 proton. The broad resonance observed centered around 8.4 ppm is a composite of narrower resonances of the H_2 protons of all eleven histidine residues as discerned by its titration behaviour. Also the little flank at 8.1 ppm shifts with the characteristics of a histidine H_2 resonance. The set of very sharp resonances centered around 7.7 ppm represents the tryptophan H_2 proton and the *ortho*, *meta* and *para* protons of the two phenylalanine residues. The resonances observed for the phenylalanine protons are very narrow in comparison to the resonance of Tyr H_3 and H_5 and the Trp H_4 and H_7 resonances, suggesting that the former are free to rotate and do not interact with the protein. Neither phenylalanine or tryptophan is present in any of the partial sequences reported for phosvitin (for review see Taborsky, 1974). Thus the assignment of the differential mobility to certain segments of the protein awaits the completion of the primary structure.

^{31}P-NMR pH titration studies. Figure 3 shows a series of ^{31}P-NMR spectra obtained for a sample of hen phosvitin at different pH values. The spectra represent all the SerP residues present in the phosvitin molecule; the broad peak arises from overlapping resonances for all these residues. All resonances shift with pH in a way characteristic of phosphomonoesters, indicating that all SerP residues are exposed to solvent. It is also readily apparent that not all phosphoryl moieties are equivalent and that many have slightly different environments and/or pH titration behaviour. The arrows in Fig. 3D indicate the resonances whose titration behaviour has been determined, in addition to that of the major resonance. These data are shown in Fig. 4. It is noteworthy that changes in shift are observed at higher pH values (Fig. 3A and 3B) which are outside of the normal pH range observed for the titration of standard solutions (Fig. 5). The titration data in Fig. 4 clearly illustrate that these inflections are observed for all resonances. As outlined before, phosvitin contains much fewer basic residues than SerP moieties, but apparently all of these SerP residues sense the presence of the basic residues leading to the high pH inflection observed for all pH titration curves. We interpret this as evidence that the salt linkages are in fast exchange between all phosphoryl moieties.

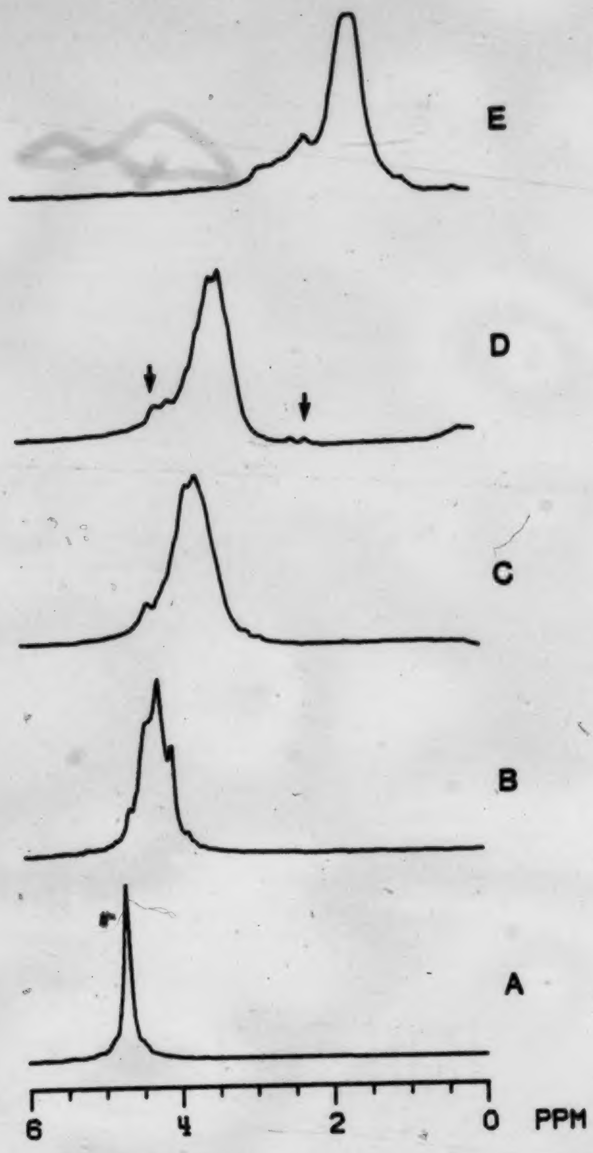

Fig. 11.3 ³¹P-NMR spectra of 1 mM phosvitin at several pH values. (A) pH 11.4; (B) pH 9.9; (C) pH 7.5; (D) pH 6.8; (E) pH 5.4. All spectra are plotted on the same scale and 200 acquisitions were collected for each spectrum.

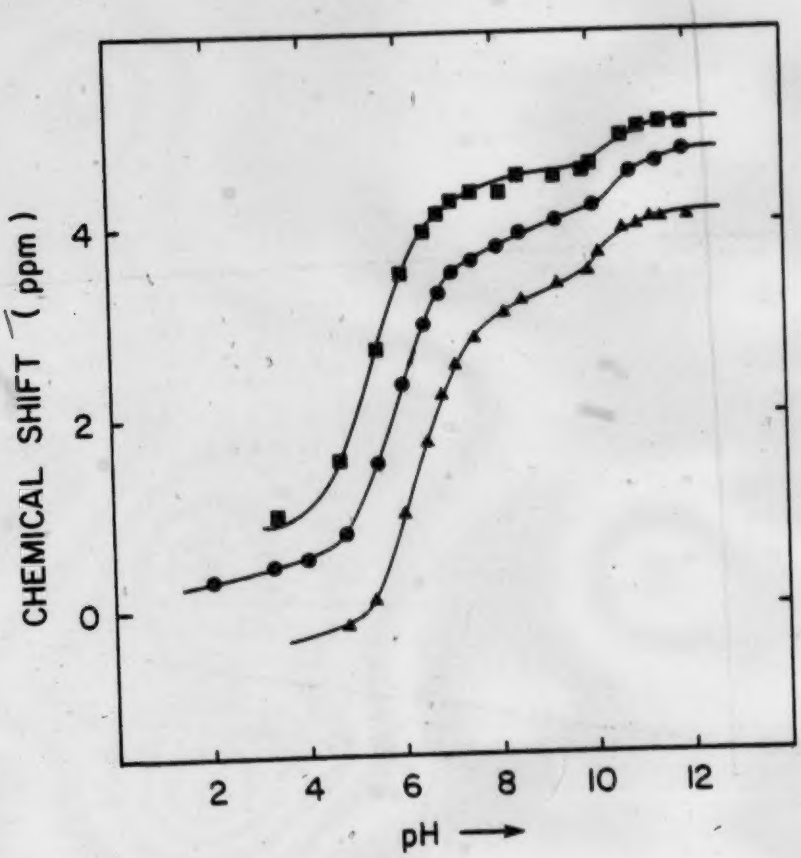

Fig. 11.4 ^{31}P-NMR pH titration curves obtained for some resolved groups of resonances in the spectra of phosvitin. (■) left resonance, (●) major resonance, (▲) right resonance.

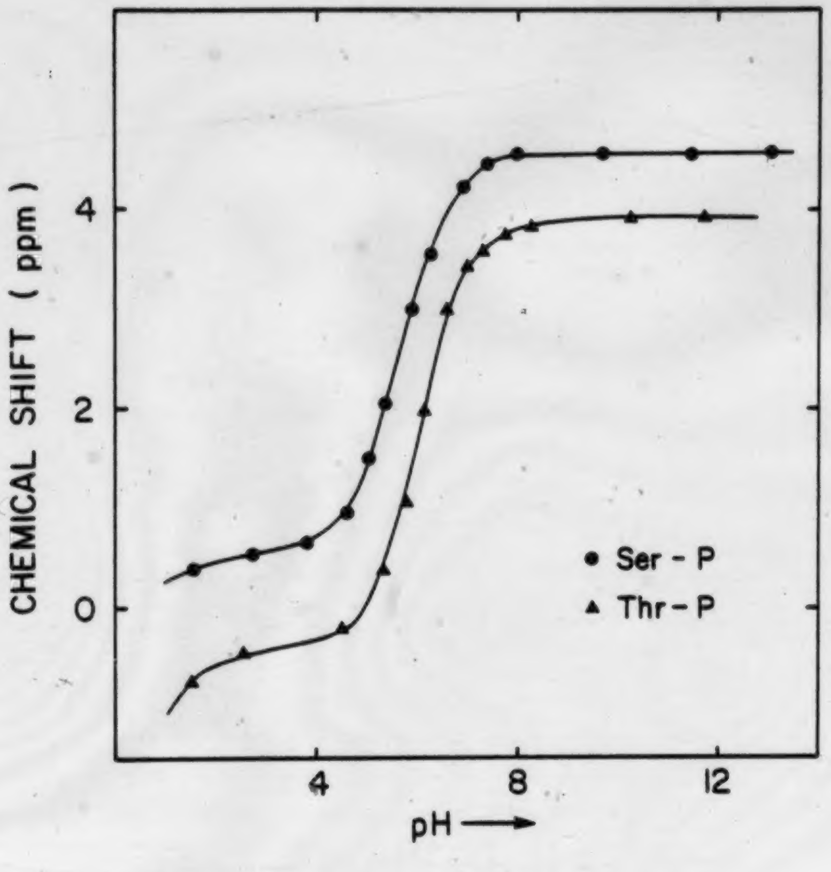

Fig. 11.5 ³¹P-NMR pH titration curves obtained for a phosphoserine and phosphothreonine standard.

The larger inflection in the titration curves observed at lower pH values corresponds to the usual titration for the conversion of the dianionic to the monoanionic species. The pKa's and Hill coefficients (n) were calculated for the lower inflections of the titration curves using a nonlinear least squares analysis program (Markley, 1975). They were pKa = 6.17, 5.79 and 5.35 and n = 0.71, 0.70 and 0.76 respectively. For a SerP standard these values are 5.75 and 0.92 respectively. Obviously, some of the protein-bound residues have pKa's different from that of the standard. However, it is noteworthy that the major peak representing at least 80% of all the residues has a pKa very similar to that of a standard. A low Hill coefficient ($n<<1$) indicates the presence of other charged groups titrating in the same pKa range (Markley, 1975). Thus the values of n = 0.70 suggest that all SerP residues have others close by that affect their titration behaviour. Of particular interest is the chemical shift position and titration behaviour of the resonance indicated by the right arrow in Fig. 3. Obviously, it bears more resemblance to a threonine phosphate than a serine phosphate residue (see Fig. 4,5). Careful amino acid analyses have in fact suggested the presence of one ThrP residue in the protein (Allerton and Perlmann, 1975). Since the intensity of this resonance suggests that it represents one residue, it is tempting to suggest that the observed resonance corresponds to this residue. Studies of proton-coupled spectra, however, have not allowed us to differentiate between a doublet proton-phosphorus splitting as would be expected for a ThrP or a triplet splitting as would be expected for a SerP residue (Ho *et al*., 1969).

Photo-laser-CIDNP ^{1}H-NMR spectra. This novel technique has been introduced recently to facilitate the detection and further study of protein bound exposed aromatic residues (Kaptein, 1978). In general, a flavin dye is added to a ^{1}H-NMR sample and the dye is excited to its triplet state in the probe of the NMR spectrometer by brief laser irradiation. The photo-excited dye can form a radical pair with accessible Trp, Tyr and His residue resulting in spin polarization accompanied by enhancement of NMR signals. These can be most readily detected by subtracting a dark from a light spectrum giving as a net result the CIDNP difference spectrum (Kaptein, 1978, 1982; Berliner and Kaptein, 1981). Since our earlier measurements indicated that phosvitin has an unfolded structure we reasoned that the flavin dye should be able to approach all His, Trp and Tyr residues present and this in turn should give rise to very intense CIDNP signals for most resonances in the aromatic part of the ^{1}H-NMR spectrum. Figure 6 shows the results obtained for a 1 mM phosvitin sample. It shows the light (Fig. 6B) and dark (Fig. 6A) ^{1}H-NMR spectrum. Several enhanced resonances can be readily detected by a comparison of the aromatic regions of both these spectra. Fig. 6C shows the light minus dark difference spectrum. The emission line

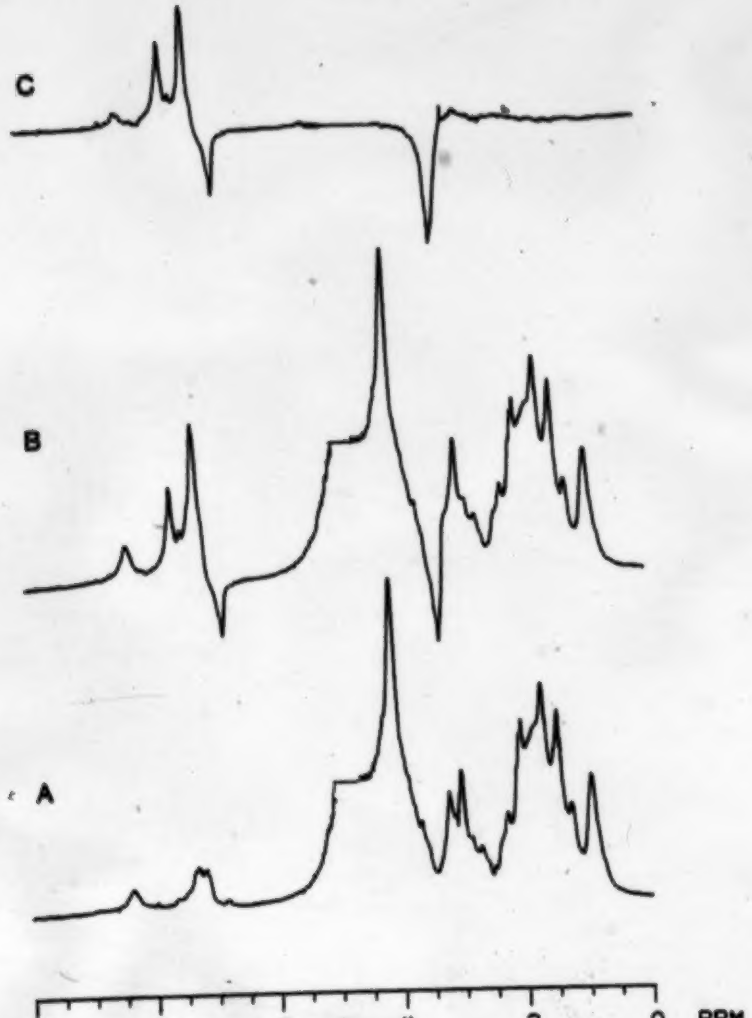

Fig. 11.6 Photo-laser-CIDNP 'H-NMR spectra of 1 mM phosvitin (pH 6.9). (A) Dark spectrum; (B) light spectrum; (C) CIDNP difference spectrum obtained by subtracting the light and dark spectra. 16 acquisitions were collected for these spectra.

at 6.80 ppm originates from the tyrosine $H_{3,5}$ protons. The strong absorptions represent the enhancements for the Trp $H_{2,4}$ (7.22 ppm) and Trp H_4 (7.61 ppm). The strong emission at 3.30 ppm represents the β-methylene protons of the Trp residue.[2] Surprisingly the His H_4 resonance at 8.35 ppm is only weakly enhanced. (For a comparison to standard CIDNP spectra see appendix 2.1 and Kaptein, 1970). The enhancements for the tyrosine and tryptophan protons are all at least a factor of 15 (compare figure 6A and 6C). This number compares favourably to those observed for standard amino acids (Kaptein, 1978; Hincke *et al.*, 1981; McCord *et al.*, 1981). However the virtual absence of any enhancement for the His protons may suggest that all eleven histidine residues are shielded from the dye.

In a further experiment we investigated the effect of the number of acquisitions on the intensities of the CIDNP effect. No relative changes in intensity were measured, but drastic changes in absolute intensity occurred, as anticipated. This is illustrated in Fig. 7. Obviously the curves do not increase linearly with the total number of acquisitions as is normally observed with other spectroscopic techniques. This deviation arises from a gradual bleaching of the dye and the fact that some modification of

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[2] A small enhancement can be detected at 7.46 ppm. This represents the Trp H_7 resonance. In free amino acids this proton is usually not enhanced; however, cross relaxation phenomena may give rise to its appearance in protein bound Trp residues (de Kanter and Kaptein, 1979).

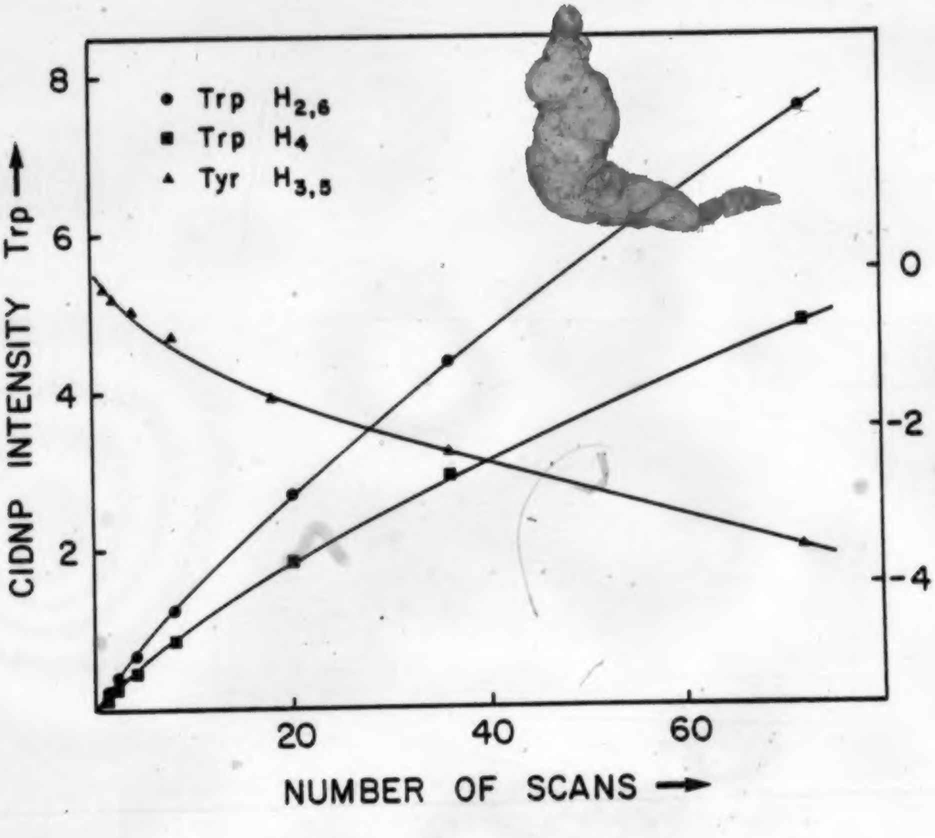

Fig. 11.7 CIDNP intensities (in arbitrary units) measured for the tryptophan and tyrosine residues of phosvitin as a function of the number of acquisitions. These data were measured for a 1 mM phosvitin sample at pH 6.9.

aromatic amino acids occurs in the course of the experiment (data not shown). A similar dependence to that shown in Fig. 7 was seen for the weakly enhanced His H_4 residues (data not shown). This confirms that the His residues were not selectively modified by photo-oxidation and thus rendered insensitive to further observation.

pH Dependence of CIDNP spectra. Since all phosphoryl moieties of phosvitin can be protonated to monoanionic species, changes in pH could bring about large differences in accessibility of the His residues. This possibility is further addressed by the set of spectra depicted in Fig. 8. The arrows indicate the CIDNP effects observed for the His H_2 (left) and His H_4 resonances (right). It appears that the His residue may become somewhat deshielded at higher pH, but the deprotonated histidine amino acid itself gives rise to more intense CIDNP effects than in the protonated form and thus the slight increase in this enhancement is more likely to be due to this effect than to a pH dependent deshielding phenomenon (see appendix 2.2, Kaptein, 1982). At the lowest pH values tested no His CIDNP effects are observed although virtually all phosphate residues should be protonated. However it could be argued again that similar behaviour is seen for the free amino acid (appendix 2.2, Kaptein, 1982). Note that the tyrosine emission has shifted and decreased in intensity at the highest pH. Both effects are caused by the titration of the phenolic hydrogen. Since proton abstraction

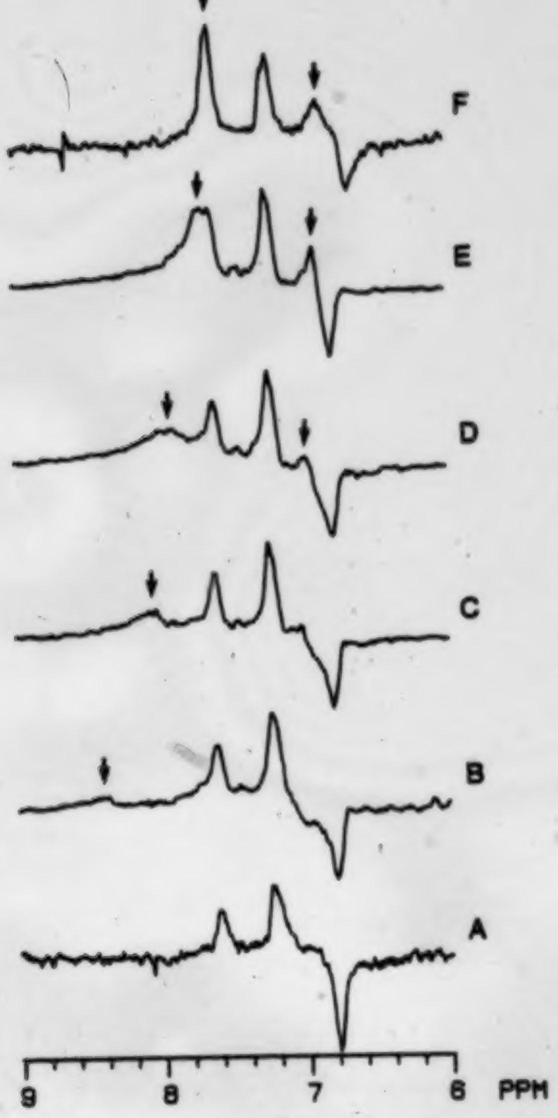

Fig. 11.8 CIDNP spectra obtained for phosvitin at different pH values. The arrows indicate the resonances observed for the histidine H_2 and H_4 resonances respectively. (A) pH 2.95; (B) pH 5.75; (C) pH 8.05; (D) pH 8.70; (E) pH 10.0; (F) pH 10.6. All spectra are plotted on the same scale.

is a necessary step in the formation of a radical pair resulting in CIDNP observed for Tyr and His residues (Kaptein, 1978, 1982), deprotonation of tyrosine residues results in a decrease of the CIDNP effect. Experiments with O-methyl tyrosine showed that this compound did not give rise to CIDNP effects. Furthermore, experiments with N-1 and N-3 methyl histidine showed that, although CIDNP was observed at low pH, these compounds at high pH did not support formation of a radical pair (data not shown). Thus proton abstraction from a histidine residue can occur at the N-1 and/or N-3 proton, and no CIDNP is observed when the methyl compounds are deprotonated at high pH. These data confirm that hydrogen abstraction is a requirement for CIDNP effects with either His or Tyr. In contrast, N-1 methyl tryptophan gives CIDNP spectra very similar to those of the normal amino acid suggesting that in this case electron transfer is the primary step to observe CIDNP effects (McCord *et al.*, 1980).

Concentration dependence of phosvitin CIDNP spectra. In the course of our studies we noticed unexpected changes in the relative intensities of the resonances observed in the CIDNP spectra. This is illustrated by the spectra presented in Fig. 9. The histidine H_2 resonance appears most prominent[3] in the spectra obtained at the lowest protein concentration and even seems to decrease at higher protein

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[3] The His H_4 resonance is also more prominent, but this one overlaps with the Trp $H_{2,4}$ resonance.

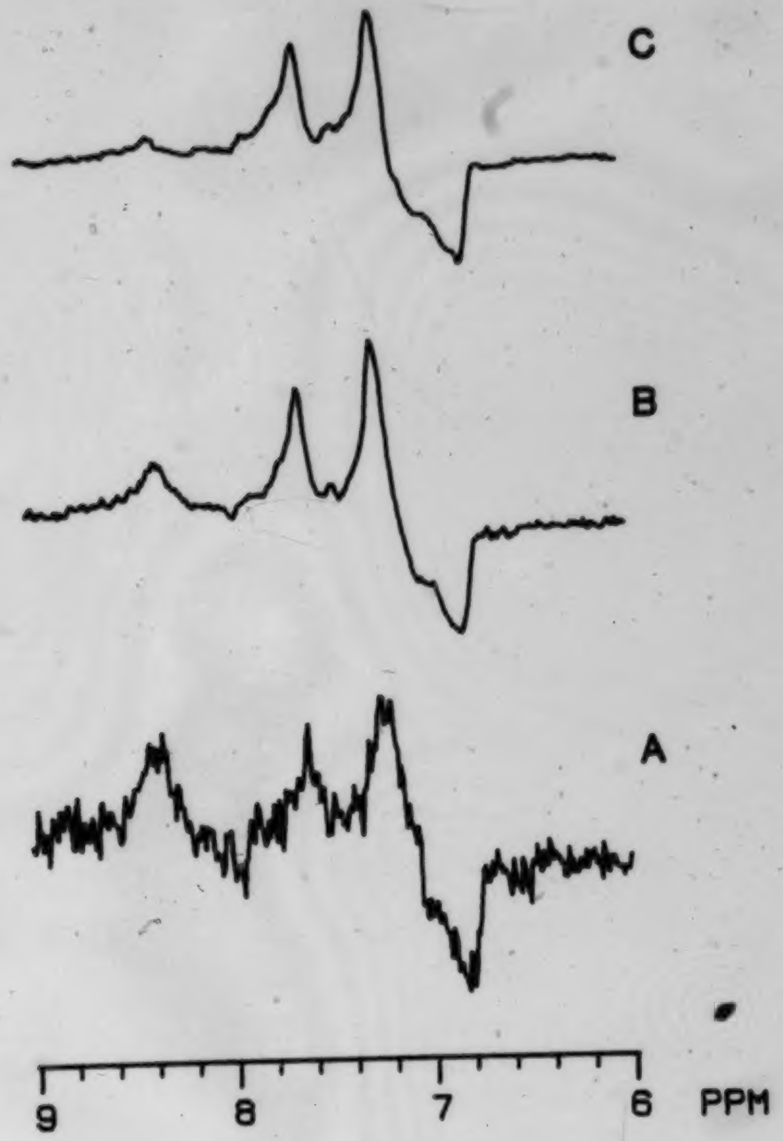

Fig. 11.9 CIDNP spectra of phosvitin obtained at different protein concentrations (pH 7.0). (A) 0.05 mM; (B) 0.25 mM; (C) 1.0 mM. All spectra are plotted on the same scale.

concentrations. This problem was addressed further by the experiment presented in Fig. 10. Clearly the intensities observed for the residues do not increase linearly with the concentration. Addition of more dye did not abolish this behaviour. Trp reaches a maximum at 0.75 mM and decreases from then on; tyrosine levels of at about 0.75 mM and the histidine intensity is always very low and decreases beyond a concentration of 0.35 mM. The behaviour of Trp was paralleled by similar effects seen for a standard. (McCord *et al.*, 1981).[4] However the CIDNP effect, determined for a His standard measured under identical conditions to those described for the experiment shown in Fig. 10, increased linearly to a concentration of about 3 mM.[4] Thus the behaviour of the His in the protein appeared anomalous. A possible explanation for this phenomenon is that Trp and Tyr can preferentially interact with the dye. Thus the increase in Trp and Tyr residues which of course occurs concomitantly with the increase in phosvitin concentration would then effectively reduce the CIDNP effect observed for the His residue. This possibility is further explored in the experiments described in the following section.

CIDNP Experiments with mixtures of amino acids.

Fig. 11 shows the data obtained for experiments with samples that all contained a constant amount of histidine (2 mM) and varying amounts of tryptophan, tyrosine or phenylalanine.

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[4] H.J. Vogel and B.D. Sykes, manuscripts in preparation.

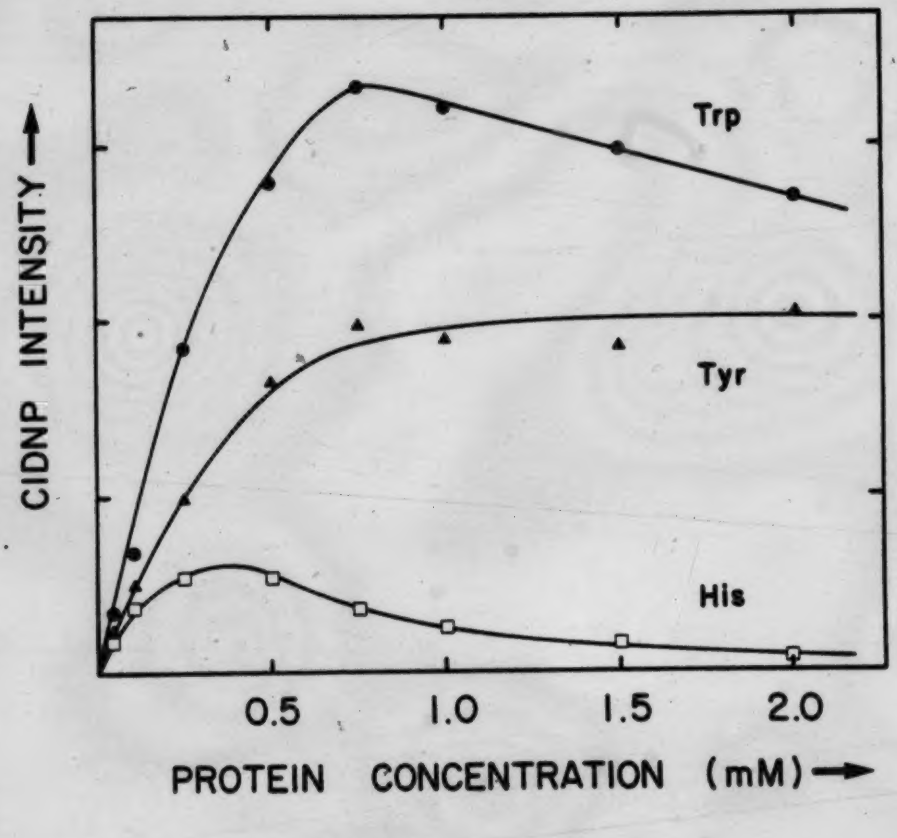

Fig. 11.10 CIDNP intensities (in arbitrary units) measured for the phosvitin, tryptophan, tyrosine and histidine residues as a function of protein concentration (pH 7.0).

The intensities measured for the His H_2 resonance in the CIDNP spectra are obviously a function of the presence of the amino acids Trp and Tyr. Both of these give strong CIDNP effects as well. Addition of Phe, which does not give rise to a CIDNP spectrum itself, does not result in a decrease. These effects of Tyr and Trp could be of competitive kinetic nature, but both residues are known to be capable of forming complexes with the dye as well (Draper and Ingraham, 1970; Isenberg and Szent-Györgyi, 1959; Fleischman and Tollin, 1965). Strict competition for available dye molecules would not explain the decrease in CIDNP effects observed for Trp at higher Trp concentrations; thus we feel that the preference of the dye for Trp and Tyr over His (see Fig. 11) originates in their binding the dye. This would also provide an explanation for the stronger effects of Trp on the intensity of the His than those of Tyr (see Fig. 11); the former is known to bind the dye more tightly (Draper and Ingraham, 1970).

These experiments provide a possible explanation for our failure to observe intense CIDNP effects for the protein-bound His residues. Moreover they also suggested the following control experiment. We reasoned that if phosvitin was entirely unfolded, its CIDNP spectrum should resemble that of a mixture of aromatic amino acids of identical composition. The spectra of Fig. 12 show a comparison between such a mixture and phosvitin. Clearly the intensity

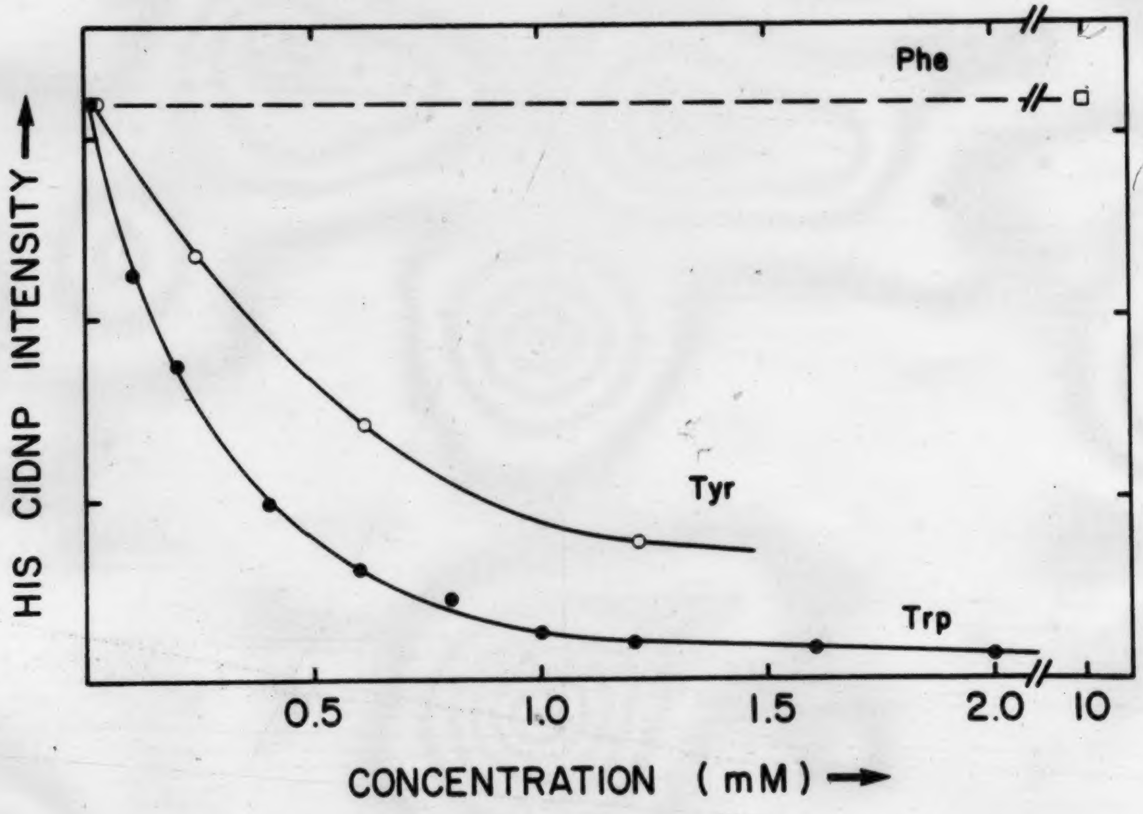

Fig. 11.11 CIDNP intensities (in arbitrary units) measured for the C_2 resonances of a 2 mM histidine solution (at pH 7.0) in the presence of varying amounts of other aromatic amino acids.

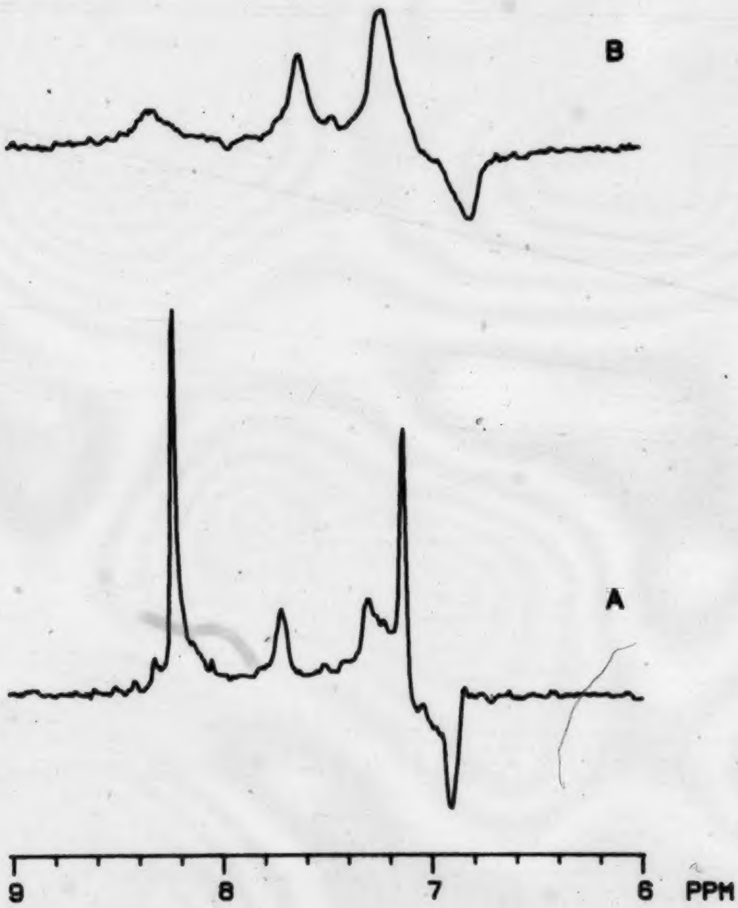

Fig. 11.12 Comparison of the CIDNP spectra obtained for a 0.25 mM phosvitin sample and a mixtuue of aromatic amino acids with a composition similar to that of the protein (pH 7.0). (A) mixture; (B) protein.

measured in the amino acid mixture for the His H_2 and H_4 resonance far exceeds the ones measured in the protein. The intensities for the Trp and Tyr appeared about equal. Thus some feature of the protein must be responsible for the low intensities measured for the His. In the foregoing we have suggested that shielding may play a role. However, as discussed in the previous section, proton abstraction is a requirement for the observation of CIDNP effects of histidine residues. Recently it was suggested that exposed surface histidine residues of ribonuclease did not give rise to CIDNP effects because of their involvement in hydrogen bonds (Bolscher *et al.*, 1979). Thus it seems more likely that the histidine residues of phosvitin are hydrogen bonded to the phosphoryl moieties then that they are shielded. If such hydrogen bonding does indeed occur, the phosvitin histidine residues are all expected to have a high pKa and a low Hill coefficient (Markley, 1975). Another possible explanation is that the protonated His residues may be in a salt linkage to the phosphoryl moieties. However, one would then expect a large deshielding to occur at higher pH. The data shown in Fig. 8 indicated that some deshielding occurs but this is most likely caused by the increase in CIDNP intensity as observed for the free amino acid (see Appendix 2.2).

^{1}H-NMR pH titration study of phosvitin. Figure 13 shows the ^{1}H-NMR titration curves obtained for the His H_2

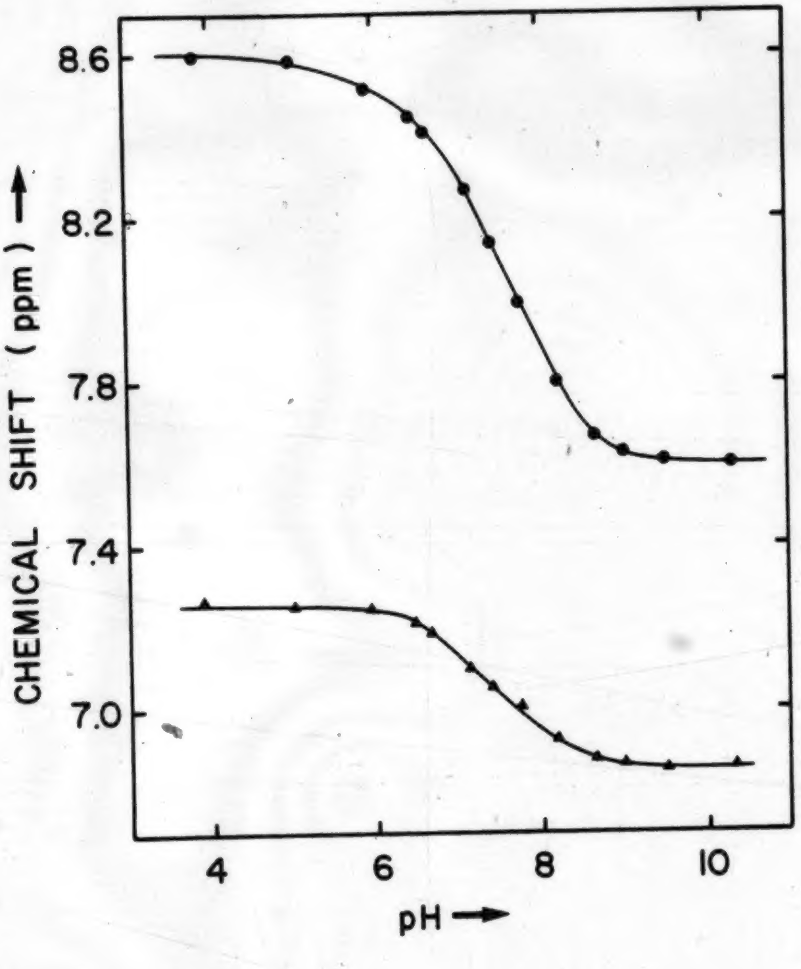

Fig. 11.13 ^{1}H-NMR pH titration curves measured for the histidine H_2 (●) and H_4 (▲) resonances of phosvitin.

and His H_4 residues respectively. In the course of these titrations some heterogeneity for the eleven histidine residues became apparent. However it was not possible to resolve any of the lines clearly and the majority behaved like the broad resonance shown in Fig. 2. The pKa's and Hill coefficients for the titration curves were calculated using nonlinear least squares analysis. They were pKa = 7.51 and 7.42 and n = 0.79 and 0.82 for the H_2 and H_4 resonances respectively. A pKa of 7.47 differs significantly from the value of 6.9 determined for the tripeptide Gly-His-Gly. Moreover, the n value of 0.80 suggests the presence of other groups in the vicinity of the protons that titrate in a similar pH range (Markley, 1975). Thus we take these data as support for our conclusion that all histidine residues of phosvitin are hydrogen bonded to the phosphoryl moieties of this protein.

Conclusions

Our studies have shown that phosvitin is a highly unfolded protein. All serine phosphate residues, all backbone amide protons, and the single tyrosine and tryptophan residues are all exposed to solvent. Although the eleven histidine residues are not available to the dye in a CIDNP experiment, all eleven can be titrated indicating their exposure to solvent. The protein is not only unfolded, but is also very flexible as was suggested by the narrow resonances observed in the ^1H-NMR and ^{31}P-NMR spectra and by our T_1 inversion recovery measurements. Some ordering is brought into all of this by the hydrogen bonding between the histidines and the SerP residues as implied by our CIDNP experiments and by the salt linkages formed between the basic amino acids and the phosphoryl moieties as inferred from the ^{31}P-NMR studies. Such linkages occur only intramolecularly since no changes in the intrinsic viscosity were detected for samples with protein concentrations between 0.2 and 2.0 mM (data not shown). A schematic representation consistent with these observations is presented in Fig. 14. It is suggested that all hydrogen bond and salt linkages can exchange rapidly between all the phosphoryl moieties within one stretch of phosphoserine residues. This exchange allows for the flexibility observed. It is of interest that the partial sequence determinations have shown that a large portion of the Arg, Lys and His residues is present in between stretches of phosphoserine residues (Belitz, 1965). Such an arrangement fits very well with our model presented in figure 14. The carboxy-terminal tyrosine is not involved in hydrogen bonding. Proton abstraction is also a requirement for the observation of CIDNP effect for this residue. If it were involved in the H-bonding it would not have shown a CIDNP spectrum, analogous to the histidines. The Trp residue may be involved in hydrogen bonding since this would not intefere with the electron transfer which results in CIDNP for this residue (McCord *et al.*, 1981; Kaptein, 1982). This model also provides a basis for

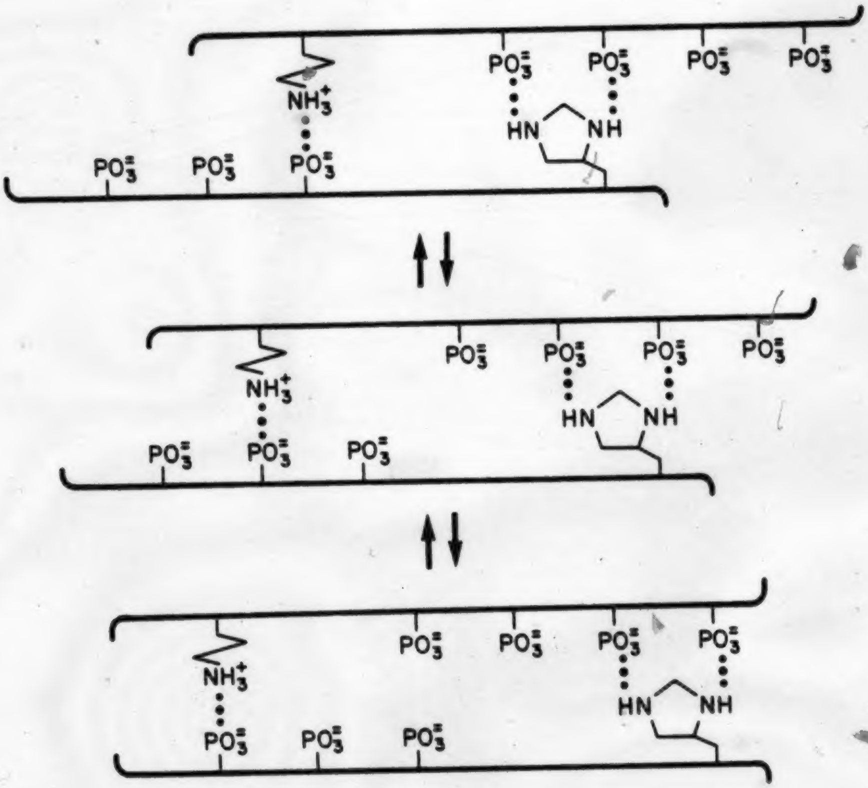

Fig. 11.14 Schematic model indicating the rapidly exchanging interactions between the basic amino acids, histidine residues and phosphoryl moieties of phosvitin.

understanding the tight complexation between Fe^{3+} and phosvitin. Most likely the metal ion can also exchange very rapidly between all different phosphoryl moieties. Such a mode of binding would explain why phosvitin is so much more effective at binding Fe^{3+} than diSerP, since this last compound does not have similar exchange possibilities. It should be mentioned here that recently similar models have been proposed for the binding of metal ions to the polyphosphate side chain of ATP and its analogues (Vogel and Bridger, 1982).

It should further be mentioned here that the results of our photo-laser-CIDNP experiments indicate that the interpretation of such spectra is not as straightforward as one would hope. On the basis of the results of the experiment shown in Fig. 11, anomalous intensity distributions can be expected for studies of all proteins. These data indicate that the intensity of a resonance is not a direct measure of its exposure, nor can changes in the intensities be directly interpreted in terms of changes in exposure or hydrogen bonding. Conclusions about aggregation (Garssen *et al.*, 1978; Berliner and Kaptein, 1980) or pH dependent conformational changes (Canioni *et al.*, 1980) may need reconsideration in view of these findings.

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CHAPTER 12

NADPH AND SUBSTRATES PROTECT ATP CITRATE LYASE FROM THERMAL AND PROTEOLYTIC INACTIVATION

Summary

Three distinct classes of compounds can effectively protect ATP-citrate lyase from thermal and proteolytic inactivation. For example, the substrate citrate is the prototype of one of these classes, in this case protecting the enzyme by binding apparently to the active site and causing a conformational change. Other compounds with suitably spaced negative charges (*e.g.*, fructose 1,6-bisphosphate, phosphoenolpyruvate) also protect, presumably by interaction with the same site. Coenzyme A, another substrate, also affords protection by binding at the active site. By testing the effects of different fragments of the coenzyme A molecule, we have shown that the major portion of this coenzyme that is recognized is the adenosine moiety. In particular, the 3'-phosphoryl group on the ribose residue of coenzyme A is an absolute requirement for binding to this site.

Further to these effects of substrates, we made the unexpected discovery that nucleotides with a 2'-phosphoryl group also afforded specific protection by interaction with a separate binding site. By far the most effective 2'-nucleotide is NADPH, which at physiological concentrations gives marked protection of ATP-citrate lyase from

degradation. Significantly, NADP$^+$ is much less effective and neither NAD$^+$ nor NADH afford any protection. Furthermore, the absence of effect of nucleotides with 2',3'-cyclic phosphates clearly distinguishes between the coenzyme A and NADPH binding sites, and points to a high degree of specificity for each.

Although all three classes of compounds interact with different sites, they appear to act by promoting an equivalent conformational change in the protein. This structural transition allows the enzyme to be proteolytically nicked, but full enzymatic activity is retained and further proteolysis is blocked. The observed effects of substrates and NADPH may play a supportive role in limiting degradation rates *in vivo*, since the level of this enzyme is under hormonal control.

Introduction

In eukaryotic organisms, fat synthesis takes place in the cytoplasm and depends upon a supply of the precursor acetyl-CoA and reducing equivalents in the form of NADPH. Since acetyl-CoA is impermiable to the mitochondrial membrane, this compound is produced in the cytoplasm by the action of ATP-citrate lyase on mitochondrially-derived citrate:

$$\text{citrate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{P} .$$

Although this step is preparatory to synthesis of both fatty acids and cholesterol, it is generally viewed that short-term regulation of lipogenesis occurs primarily at the level of acetyl-CoA carboxylase [1] and 3-hydroxy-3-methylglutaryl-CoA reductase [2], respectively. However, there has been a recent renewal of interest in the possibility that regulation of ATP-citrate lyase may be a contributing factor to the control of lipogenesis. For example, it has been shown that this enzyme may be phosphorylated both by cyclic-AMP dependent protein kinase, in response to glucagon, and by an insulin-stimulated, cyclic-AMP independent protein kinase [3-5]. The regulatory or catalytic consequences of such structural phosphorylations are not yet apparent. Neither the kinetic properties of the enzyme nor its stability are affected by phosphorylation, for example [6]. It is possible that phosphorylation may be involved in regulation of association of the enzyme with the mitochondrial membrane [7] or control of its intracellular degradation [8].

While it has long been recognized that the synthesis of this enzyme is under hormonal control [9], recent experiments in this laboratory have suggested that hormonal or nutritional influences may affect the susceptibility of ATP-citrate lyase to degradation, perhaps through the action of an incompletely-characterized, low molecular weight, "stabilizing factor" [10] which is known to exert similar effects on phosphofructokinase [11].

In this paper, we describe experiments that delineate the effects of various compounds on the stability of ATP-citrate lyase and on its susceptibility to proteolytic attack, a property that generally correlates with turnover rates observed *in vivo* [12]. The results show that ATP-citrate lyase is strongly stabilized by some of its substrates and products. Moreover, our studies indicate that the enzyme has a site, apparently distinct from the active center, for high-affinity and specific binding of NADPH, the occupancy of which results in a form of enzyme that is dramatically protected from proteolysis. This last observation is particularly intriguing, since it may provide one basis for the coordination of the production of acetyl-CoA for lipogenesis with the availability of reducing equivalents for this process.

Experimental Procedures

Enzyme preparations. ATP-citrate lyase was purified to homogeneity from livers of male Sprague-Dawley rats that had been starved for 24 hr and refed for 48 hr on a diet containing 63% sucrose, 24% casein, 2.5% cellulose, plus salt and vitamin mixture. The purification procedure was as described by Osterlund *et al*. [10], modified in later batches to include the addition of 1% rabbit serum to the liver homogenate to avoid proteolytic nicking during the isolation of the enzyme [13]. The enzyme purified in this way had a specific activity of 8.5 units/mg and gave rise to one band (mol wt 120,000) on SDS-polyacrylamide gel electrophoresis. ATP-citrate lyase prepared by this procedure is expected to be phosphorylated, presumably at the insulin-stimulated site [14,15]. Consistent with this is our observation of a broad phosphoserine peak by ^{31}P-NMR spectroscopy (H.J. Vogel and W.A. Bridger, unpublished) and by the fact that the enzyme would incorporate ^{32}P from labeled ATP in the presence of cAMP-dependent protein kinase.

Other materials. ATP-citrate lyase stabilizing factor was prepared as previously described [10]. Guanosine-5'-diphosphate-3'-monophosphate, 2'-deoxyadenosine-3',5'-diphosphate and adenosine-2',5'-diphosphate were obtained from P.L. Biochemicals. Other nucleotides were purchased from Boehringer-Mannheim Canada or from Sigma Chemical Company. Fructose-2,6-diphosphate was synthesized according to Van Schaftingen *et al*. [16]. Equimolar solutions of adenine nucleotides and nicotinamide nucleotides were prepared either by weight, or by treatment of solutions of NADP$^+$ or NADPH with nucleotide pyrophosphatase (Sigma). In the latter case, completion of the reaction was confirmed by the ^{31}P-NMR spectrum of the products, where the diphosphodiester

(-10 ppm) and phosphomonoester (+4 ppm) bonds are well resolved. TPCK-trypsin (trypsin treated with tosyl-phenylalanine chloromethylketone to inactivate chymotrypsin) was obtained from Worthington, and other proteases and inhibitors were from Sigma.

Thermal inactivation of ATP-citrate lyase. The rate of inactivation of the enzyme at 37° was measured as described by Osterlund *et al*. [10]. In latter experiments, when preparations of ATP-citrate lyase avoided proteolytic nicking (see above), it was found that the enzyme was much less susceptible to heat denaturation. In this case, the enzyme was deliberately nicked with trypsin at 25° [17] prior to assay for thermal inactivation.

Proteolytic inactivation of ATP-citrate lyase. For assay of inactivation by proteases, the ATP-citrate lyase was incubated at 37° for timed intervals in the presence of the proteolytic enzyme (1% w/w). For assay of the rate of nicking by trypsin, ATP-citrate lyase was incubated with 0.2% trypsin at 37°; the products were analyzed by densitometric scanning of stained SDS-gel electrophoretic patterns of the digests.

Assay of ATP-citrate lyase activity. Enzyme activity was measured by the coupled procedure previously described [18].

Results

Stabilizing effect of NADPH. Previous reports from this laboratory [10,18] described protection of ATP-citrate lyase from thermal inactivation by a stabilizing factor purified from rat liver; the chemical nature of this factor is still not precisely known. In the course of attempts to identify this factor, we have discovered that a variety of classes of compounds can exert striking protective effects on this enzyme. Because of possible implications for regulation of lipogenesis, we are particularly intrigued by our observation that NADPH, the coenzyme that supplies reducing equivalents for fatty acid biosynthesis, exerts a strong and specific stabilizing effect on ATP-citrate lyase. Fig.1 illustrates protection of ATP-citrate lyase by NADPH from thermal inactivation. The NADPH-binding site appears to be quite specific for the reduced coenzyme, since $NADP^+$ proved much less effective. Remarkably, neither NADH nor NAD^+ afforded significant protection at the concentrations tested.' It has been noted earlier [10] that the substrates CoA and citrate are also very potent stabilizers of ATP-citrate lyase. Equivalent experiments to that shown for NADPH in Fig. 1 showed that the concentrations of these compounds for half-maximal protection are 1 μM for CoA and 4 mM for citrate. These values are close to reported Km's for

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'It is noteworthy that Hoffmann *et al*. [19] have reported that ATP-citrate lyase may be displaced from a column of Blue Sepharose by $NADP^+$ but not by NAD^+.

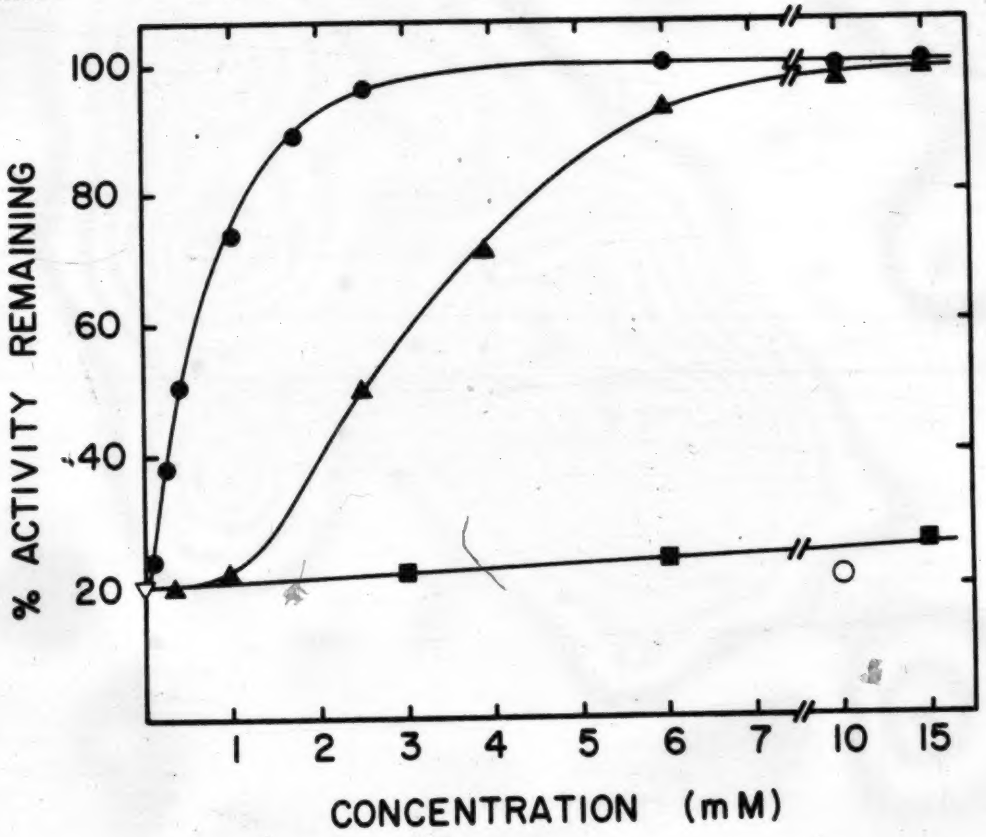

Fig. 12.1 Protection of ATP-citrate lyase from thermal inactivation by NADPH and NADP⁺. The data show the enzyme activity remaining after 30 min at 37° and pH 8.3 in the presence of the indicated concentrations of the following: (●-●): NADPH; (▲-▲): NADP⁺; (■-■): NADH; (○-○): NAD⁺; (▽), control.

these substrates [20], suggesting that the stabilizing effects of CoA and citrate are due to their interaction with the active site. Evidence to the effect that NADPH interacts at a separate site will be considered in the following section.

Mapping the nucleotide binding site. The purpose of these experiments was to demonstrate that the active site and the NADPH site are distinct, and to probe the specific structural requirements for binding of compounds to these sites. In the process, we have explored possible effects of other metabolites involved in lipid biosynthesis. The results are summarized in Table 1. As the organization of this table implies, the results indicate that ATP-citrate lyase possesses three distinguishable binding sites whose occupancy results in greatly enhanced stability. Citrate, for example, a substrate for ATP-citrate lyase, effectively protects the enzyme from thermal inactivation. More interesting, however, is the spectrum of results observed with coenzymes and nucleotides possessing either a 3'- or 2'-phosphoryl group. The parent compound of the group containing a 3'-phosphoryl is the substrate CoA, which is capable of giving 50% of full protection at a concentration of 1 μM. Acetyl-CoA (a product of the reaction catalyzed by ATP-citrate lyase) is somewhat less effective. 3'5'-ADP (*i.e.* CoA without the phosphopantotheine residue) is also effective, albeit at higher concentrations, while 3'-AMP is

Table 12.1

Effects of various compounds on the stability of ATP-citrate lyase

A. Compounds that protect (conc. given is that providing 50% protection)

Site 1

Compound	Conc.
β-NADPH	0.25 mM
β-NADP+	3 mM
2',5'-ADP	2.5 mM
2'-AMP	20 mM
2'-GMP	40 mM
α-NADPH	<0.25 mM
α-NADP+	2.5 mM
2',5' ADP + NMNH	2.5 mM

Site 2

Compound	Conc.
CoA	1 μM
Acetyl-CoA	5 μM
3'-NADP+	25 μM
3'-AMP	8 mM
3',5' ADP	0.1 mM
Etheno CoA	0.1 mM
2'-deoxy-3',5' ADP	<0.1 mM

Site 3

Compound	Conc.
Citrate	4 mM
Fructose-1,6-bisphosphate	6 mM
Phosphoenolpyruvate	5 mM

B. Compounds that do not protect (conc. given is highest tested)

Compound	Conc.
Nicotinamide	10 mM
NMNH	10 mM
NMN	10 mM
Ribose-5-phos.	10 mM
Nicotinamide-hypoxanthine-DPH	10 mM
AMP	10 mM
ADP-ribose	10 mM
NAD+	10 mM
NADH	10 mM
2',3'-cyclic AMP	10 mM
2',3'-cyclic NADP+	10 mM
3',5'-cyclic AMP	10 mM
Pyrophosphate	10 mM

Compound	Conc.
Dephospho CoA	1 mM
Pantothenate	10 mM
Ribose-5-phos.	10 mM
AMP	10 mM
ADP-ribose	10 mM
3'-GMP	40 mM
5'-PP-G-3'P	2 mM
2',3'-cyclic AMP	10 mM
2',3'-cyclic NADP+	10 mM
3',5'-cyclic AMP	10 mM

Compound	Conc.
Fructose-6-phos	10 mM
6-phosphogluconate	10 mM
Fructose-2,6-bisphosphate	0.1 mM
α-glycerol phosphate	10 mM

only weakly protective. Of the group of compounds possessing a 2'-phosphoryl group, NADPH affords the most dramatic stabilization. $NADP^+$ is much less effective, no more so than 2',5'-ADP (*i.e.*, $NADP^+$ without the NMN reseidue). 2'-ADP is only weakly protective. Curiously, the glycosidic analogues of $NADP^+$ and NADPH are about as effective as the natural anomers. This site is less tolerant of change at the adenine end of the coenzyme molecule; the hypoxanthine analogue of NADPH is ineffective.

It is particularly intriguing that the $NADP^+$ analogue, 3'-$NADP^+$, affords full protection at a concentration below 50 μM, suggesting that it may interact with the CoA-binding site rather than the NADPH site. Significantly, neither 10 mM 2',3'-cyclic $NADP^+$ nor 10 mM 2',3'-cyclic AMP gave measurable protection. This is in strong support for our view that the enzyme possesses two sites with specificity for 2'- or 3'-phosphoryl substituents on a coenzyme structure, respectively. Furthermore, since the enzyme distinguishes these compounds from its substrates ADP and ATP (neither of which cause significant stabilization), separate binding sites exist for specific recognition of nucleotides with or without phosphoryl groups on the 2' or 3' positions of the ribosyl moiety. It is recognized, of course, that failure to protect does not necessarily prove that a compound does not bind to a stabilizing site, since one could visualize non-productive binding without accompanying change in enzyme structure.

The CoA-binding site. Our data suggest that the most important structural feature for interaction with this site is the presence of a 3'-phosphoryl group on a nucleotide. This is most clearly shown by the failure of dephospho-CoA to produce stabilization at concentrations 1000 times that which is effective for CoA. The adjacent 2'-hydroxyl group is not important, since 3'5'-ADP and 2'-deoxy-3'5'ADP are about equally effective. Interestingly, this site can tolerate modification of the base, as shown by the effect of the etheno derivative of CoA, but analogues containing guanine are rejected, suggesting that the 2-amino group is incompatible with productive binding.

The citrate site. Relatively simple compounds can provide significant protection provided that they contain two or more negatively charged groups at appropriate distances, as might be expected for interaction with a site designed to recognize citrate. Thus, phosphoenolpyruvate and fructose-1,6-bisphosphate protect, albeit at above expected physiological concentrations, while fructose-6-phosphate and the lipid precursor -glycerol phosphate, for example, do not. Significantly, two compounds that have been reported to activate phosphofructokinase, namely fructose-2,6-bisphosphate [16,21,22] and 6-phosphogluconate [23], do not cause significant protection, at least at concentrations that are effective on phosphofructokinase.

Protection against proteolytic inactivation: Productive occupation of any of the three sites described above leads to protection against inactivation by a variety of proteolytic enzymes. As will be seen, this is accomplished by inducing the enzyme to adopt a conformation that is still able to be nicked, but following which it is both fully active and resistant to further proteolytic attack.

As noted earlier, preparations of ATP-citrate lyase that have not been proteolytically nicked are insensitive to thermal inactivation under the conditions of the foregoing experiments. Since intracellular protein turnover must involve proteolytic attack, we have investigated the effects of various compounds on the susceptibility of the enzyme to a variety of proteases. Two aspects of proteolysis of ATP-citrate lyase need to be considered, namely the rate of nicking of the enzyme, known to result in production of a form that retains full activity [17], and subsequent inactivation by further proteolysis. In agreement with Singh *et al*. [17], we have found that treatment with trypsin at 25° results in controlled production of the nicked species, without inactivation. Trypsin attack at 37°, however, causes further proteolysis accompanied by inactivation. Fig. 2 shows the effects of various compounds on the rate of nicking of the enzyme by 0.2% trypsin (*i.e.*, the rate of disappearance of the native subunit of molecular weight 120,000). While citrate, CoA and NADPH do afford some

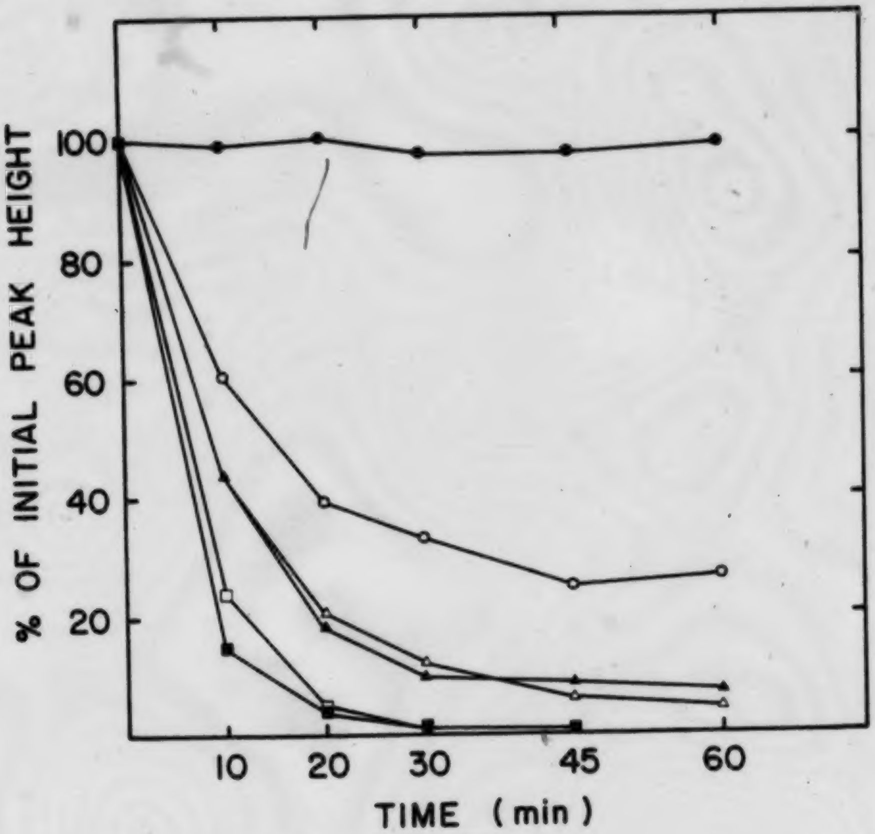

Fig. 12.2 Effect of various compounds on the rate of nicking of ATP-citrate lyase. ATP-citrate lyase (0.3 mg/ml) was incubated in 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol at 37° in the presence of trypsin (0.2%) and the additions indicated below. At time intervals, 50 μl samples were removed to which were added 1 μg soybean trypsin inhibitor. The samples were then subjected to SDS-polyacrylamide gel electrophoresis, and the stained gels were densitometrically scanned. The data shown in the figure indicate the height of the peak corresponding to the intact 120,000 mol. wt. subunit. The additions are as follows: (■-■): none; (○-○): 10 mM citrate; (▲-▲): 0.6 mM CoA; (Δ-Δ): 6 mM NADPH; (□-□): stabilizing factor (1 mg/ml); (•-•): control (trypsin inhibitor added at zero time). Plots were also made (data not shown) for the increase in the two major products of the proteolytic nicking (mol. wts appoximately 60,000 and 70,000). These showed the expected reciprocal relationship to the disappearance of the intact species.

protection against nicking, little of the intact enzyme remains after 1 hr under these conditions, although in all cases the enzyme retains full catalytic activity. The effect of these compounds on inactivation is more significant, however, when tested with a five-fold elevated concentration of trypsin. The results of Fig. 3 show that NADPH, citrate and CoA provide virtually complete protection against inactivation by trypsin. Stabilizing factor is less effective, at a concentration found to be saturating for its other effects [10]. Qualititatively similar results were obtained with pronase (Fig. 4), subtilisin (Fig. 5) and chymotrypsin (Fig. 6). Of these, attack by subtilisin is least responsive to protection, while inactivation by chymotrypsin provides the most dramatic demonstration of protection by NADPH, citrate and CoA. Thus, there is a clear correlation between the ability to confer thermal stability upon nicked ATP-citrate lyase and the ability to protect the enzyme against inactivation by proteases. Inactivation by chymotrypsin was therefore adopted as an additional probe for the detailed evaluation of the structural requirements for binding of coenzymes and nucleotides to the respective sites on ATP-citrate lyase (see Table 1).

The SDS-gel electrophoresis patterns shown in Figure 7 show that the protective compounds such as NADPH and CoA do not affect primarily the initial nicking of the molecule to form the active aggregate of 68,000 and 57,000 mol wt

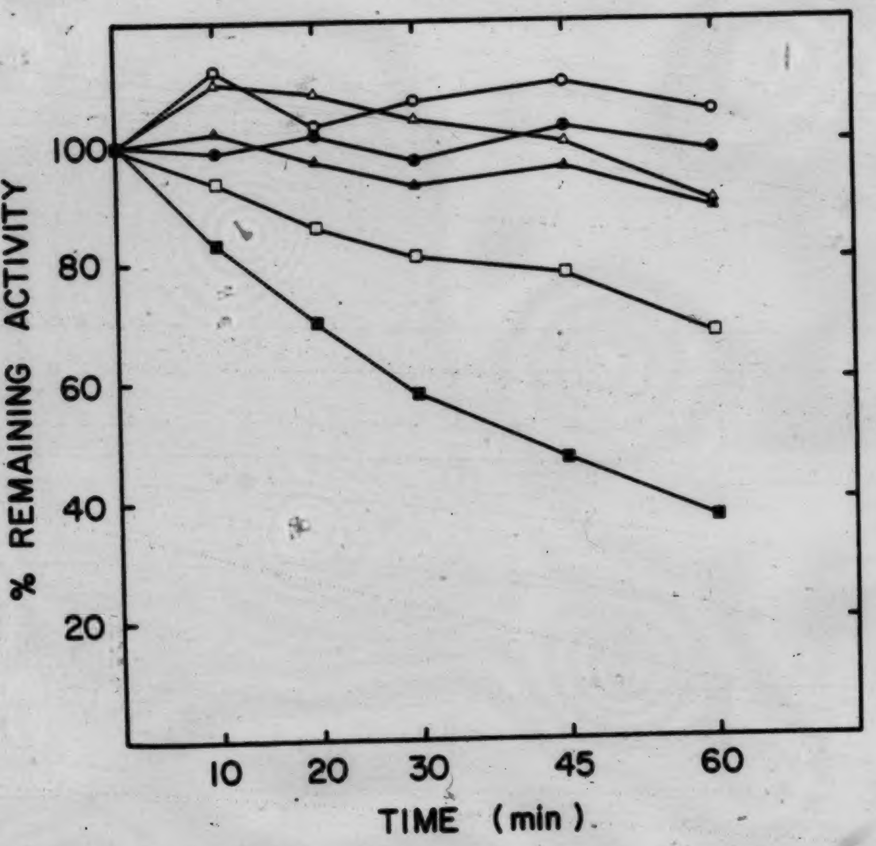

Fig. 12.3 Protection of ATP-citrate lyase from inactivation by trypsin. ATP-citrate lyase was incubated at 37° in 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and 1% trypsin solution, with the following additions: (○-○): 10 mM citrate; (▲-▲): 0.6 mM CoA; (△-△): 6 mM NADPH; (□-□): stabilizing factor (1 mg/ml); (■-■): control (no additions); (●-●): soybean trypsin inhibitor (5 *u*g).

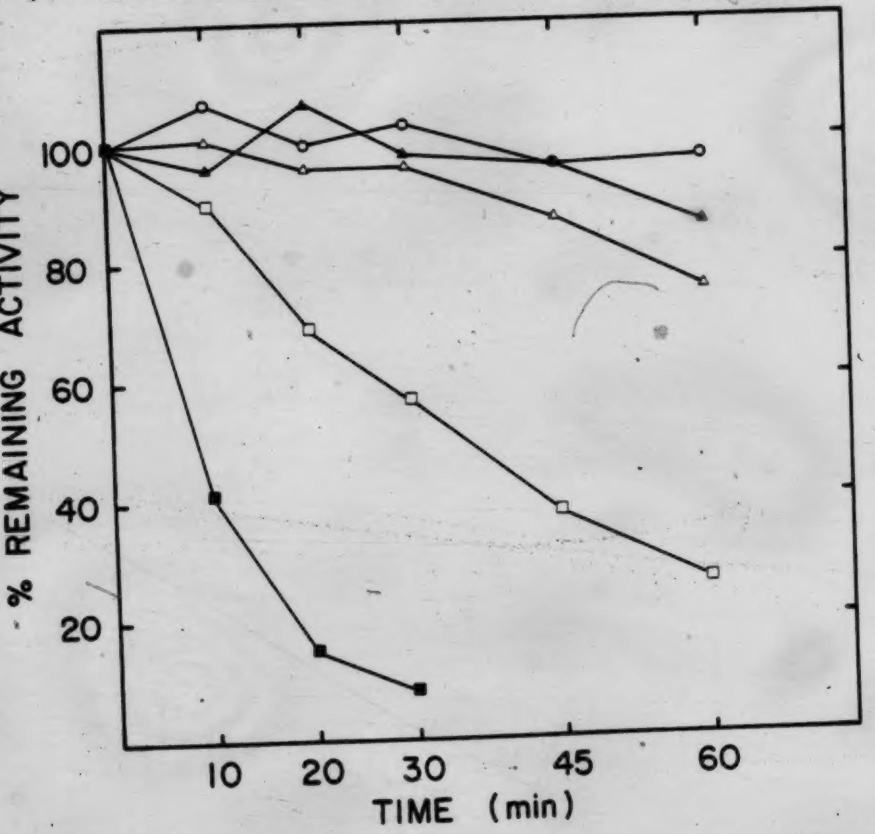

Fig. 12.4 Protection of ATP-citrate lyase from inactivation by pronase. ATP-citrate lyase was incubated at 37° in 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and 1% pronase solution, with the following additions: (o-o): 10 mM citrate; (▲-▲): 0.6 mM CoA; (Δ-Δ): 6 mM NADPH; (□-□): stabilizing factor (1 mg/ml); (■-■): control (no additions).

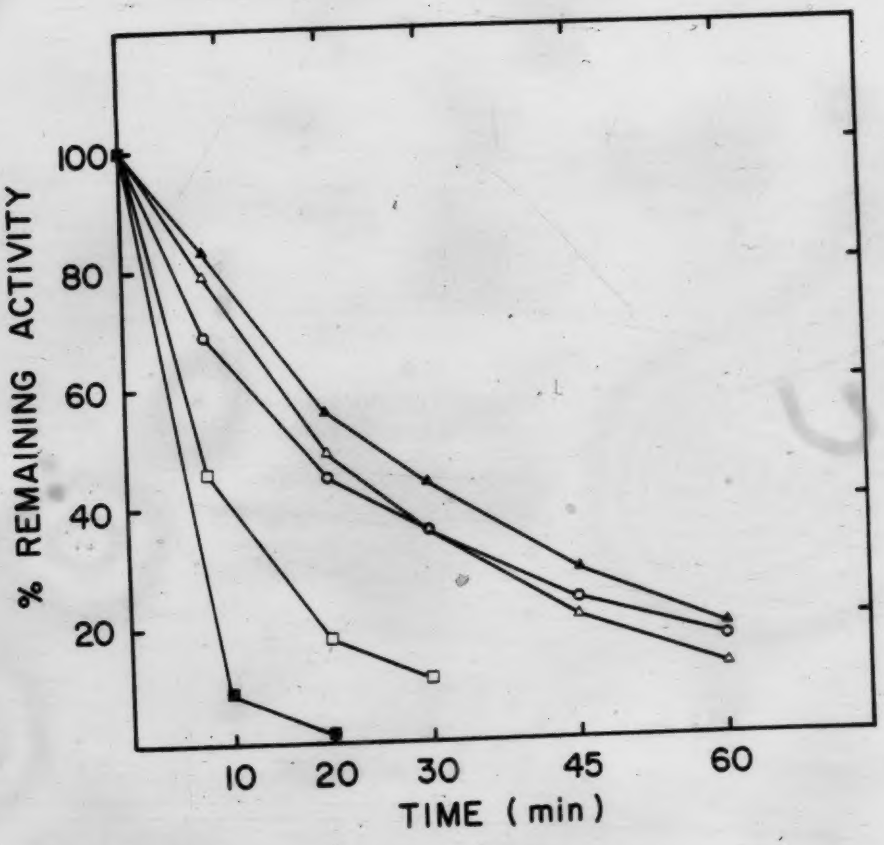

Fig. 12.5 Protection of ATP-citrate lyase from inactivation by subtilisin. ATP-citrate lyase was incubated at 37° in 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and 1% subtilisin solution, with the following additions: (o-o): 10 mM citrate; (▲-▲): 0.6 mM CoA; (Δ-Δ): 6 mM NADPH; (□-□): stabilizing factor (1 mg/ml); (■-■): control (no additions).

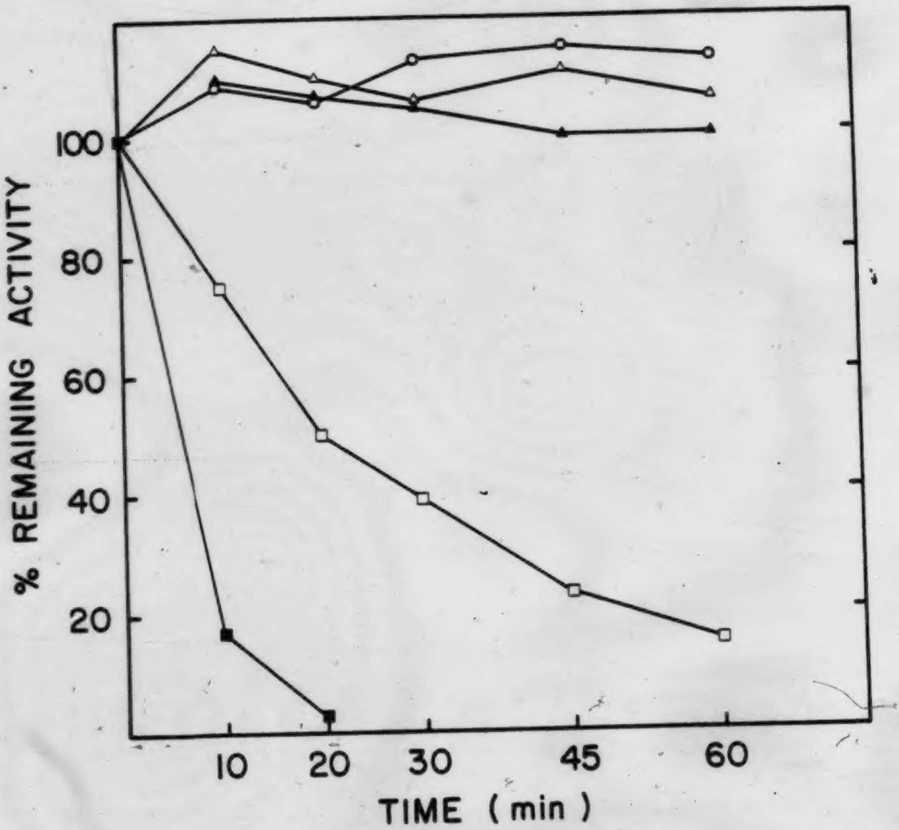

Fig. 12.6 Protection of ATP-citrate lyase from inactivation by chymotrypsin. ATP-citrate lyase was incubated at 37° in 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and 1% chymotrypsin solution, with the following additions: (o-o): 10 mM citrate; (▲-▲): 0.6 mM CoA; (Δ-Δ): 6 mM NADPH; (□-□): stabilizing factor (1 mg/ml); (■-■): control (no additions).

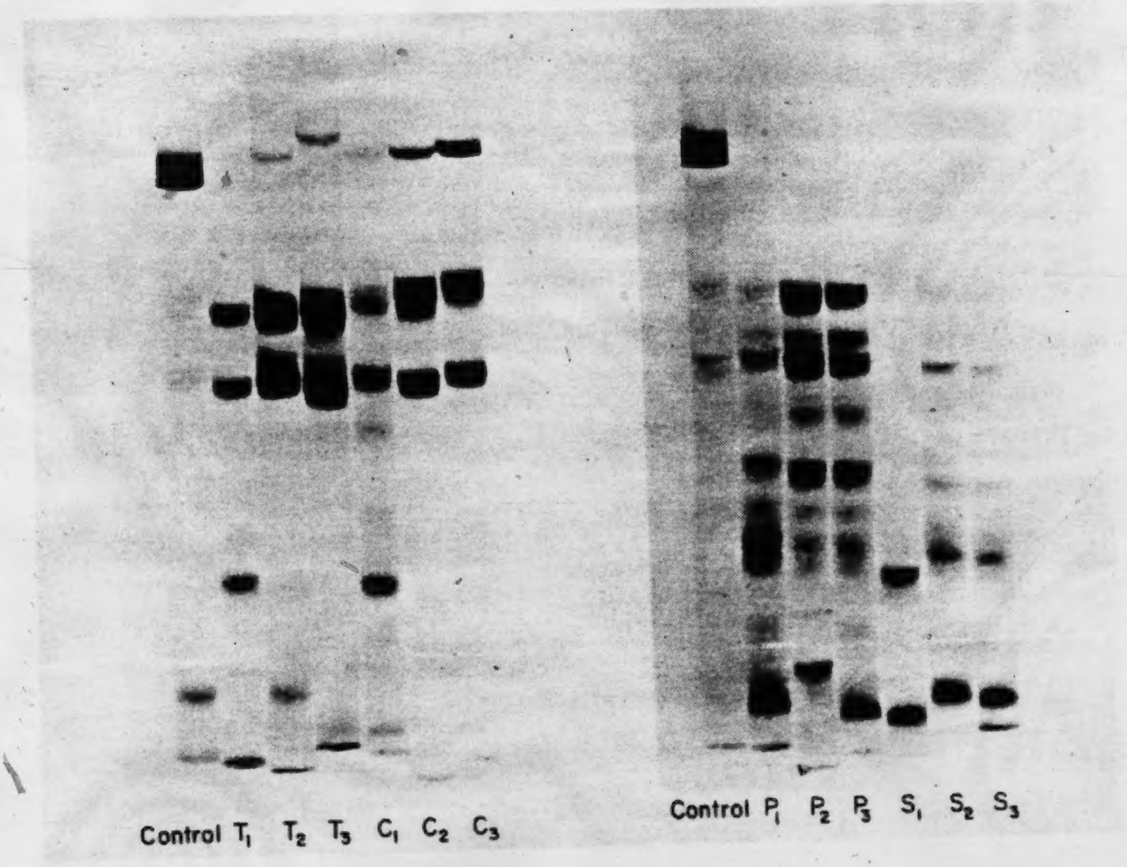

Fig. 12.7 Effect of NADPH and CoA on the nicking pattern of ATP-citrate lyase by proteases. The treatments were as described in the legends to Figs 3-6, except that in all cases the protease concentration was 0.5%. Samples were removed after 30 min at 37° and were immediately boiled in 1% SDS solution before electrophoresis on 7% polyacrylamide. The control samples contained no protease. The other samples are identified as follows: T, C, P and S indicate treatment with trypsin, chymotrypsin, pronase and subtilisin, respectively. The subscripts *1* indicates that no further additions were made, while the subscripts *2* and *3* indicate the presence of 0.6 mM CoA and 6 mM NADPH, respectively, in the incubation mixture.

fragments; instead, subsequent degradation of the two large polypeptides is blocked. For example, digestion with trypsin or chymotrypsin in the presence of NADPH or CoA proceeds only to this stage, with retention of full catalytic activity. Virtually identical results were obtained with citrate (data not shown), supporting our view that occupation of any of the three sites is sufficient for production of an equivalent conformation. Similarly, pronase treatment results in accumulation of the nicked species. Digestion with subtilisin, on the other hand, is less sensitive to the presence of NADPH or CoA, with considerable production of small fragments accompanied by inactivation. This protease appears to attack ATP-citrate lyase differently, with little build-up of the nicked intermediate.

Discussion

Clearly, glycolysis and lipogenesis in the liver are coordinately controlled at not less than three levels, namely control of enzyme concentration (by regulation of synthesis and perhaps degradation), covalent control such as phosphorylation by hormone-sensitive protein kinases, and short-term control by a variety of low molecular weight effectors. Many details remain clouded, and the rank of importance of these effects is still not clear. In the area of lipogenesis, there have been several recent suggestions of allosteric control of different enzymes, such as acetyl-CoA inhibition of $NADP^+$-malic enzyme [24], CoA inhibition of acetyl-CoA carboxylase [25], together with the various effects on phosphofructokinase mentioned earlier. Furthermore, Gottschalk and Kemp [26] have recently reported that NADPH and other dinucleotides can affect the activity of muscle phosphofructokinase by competing with cAMP for the allosteric activating site; an equivalent effect on the liver enzyme remains to be demonstrated. It seems unlikely that any one of these effects could play an overriding role in regulation of lipid biosynthesis, but each could contribute and perhaps interplay with other controls such as the effects of phosphorylation and fructose-2,6-diphosphate on phosphofructokinase, for example.

Our observation that ATP-citrate lyase possesses a specific site for binding physiological concentrations of NADPH raises the question of a possible regulatory role for NADPH on the activity of the enzyme. One could visualize, for example, that capacity for lipogensis might be made sensitive to the redox state of the cell in order to coordinate fat synthesis to the availability of carbohydrate precursors. This possibility would be strengthened if there were evidence for substantial swings in the NADPH/NADP$^+$ ratio in response to various nutritional or hormonal effectors. Indirect measurements made by Veech *et al.* [27] suggested that this ratio is somewhat sensitive to these stimuli but that the changes in NADPH concentration are not sufficiently large for it to act as an important signal for metabolic control. Nevertheless, our results are consistent with the idea that a drop in the NADPH concentration, such as that expected during diabetes or carbohydrate starvation, should make ATP-citrate lyase a more susceptible substrate for intracellular proteolysis and turnover. Aside from possible effects on turnover, NADPH could influence the association of ATP-citrate lyase with mitochondrial membranes, a concept that is made especially credible in view of the demonstration by Janski *et al*. [7] that the substrates CoA and citrate accelerate the release of the enzyme from isolated mitochondria. Since we believe that NADPH mimics the effects of these substrates on the enzyme's conformation, it may also have a similar influence on its affinity for the mitochondrial membrane. In this context, it is noteworthy that the concentration of citrate does not change significantly during lipogenic conditions [28], while the level of CoA does show a modest increase [29].

We are impressed by the high degree of specificity that ATP-citrate lyase displays in distinguishing nucleotides bearing 2'- or 3'-phosphoryl groups, while rejecting those with 2',3'-cyclic phoshates. High selectivity for NAD$^+$ over NADP$^+$ is achieved by lactate dehydrogenase, for example, by interaction of an enzymic carboxylate with the 2'-OH of the coenzyme [30]. Arginine residues likely contribute positively to the recognition, in a way such as that implied by the protection of fatty acid synthetase from phenylglyoxal by $NADP^+$ and analogues containing a 2'-phosphoryl group [31]. The specificity of ATP-citrate lyase indicates the presence of precisely positioned charged groups in the regions that bind the ribose residues of the nucleotides, allowing the enzyme to distinguish reliably between these ligands.

Note added in proof. Subsequent kinetic experiments have shown that NADPH (or 2'5'ADP) does not interfere with the binding of CoA, supporting our suggestion that the enzyme has separate binding sites for these two nucleotides. However fructose 1,6 di-phosphate (FDP) nor PEP inhibited competitively with citrate. Thus it is unlikely that these two exert their protective effect by binding to the citrate-binding site as we suggested. Although they bind to a separate site, both have the same effect on the enzymes conformation as we could show by performing the experiment of Fig. 7 in the presence of these two metabolites (data not shown). The only effect that saturating amounts of 2'5'ADP, FDP and PEP seemed to exert on the kinetics of the enzyme was a very small decrease in the Km for ATP. This effect was reproducible but did not seem statistically significant. Since this effect was so small we did not investigate the possibility if the effects of these three activators were additive. Therefore we do not know at present whether all three ligands act by binding to the same site or if each one has its own binding site.

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CHAPTER 13

DISCUSSION AND CONCLUSIONS

In this final chapter we will try to discuss our findings as outlined in Chapters 2-12 with respect to the questions brought forward in Chapter 1. These were, in short: What can we learn about the analysis of ^{31}P-NMR spectra of phosphoproteins, and what can we learn about phosphoryl-transferring enzymes and regulatory phosphorylation sites? These questions will be considered in turn in the following.

13.3 Interpretation of the chemical shifts measured in ^{31}P-NMR spectra.

In studies of *in vivo* metabolism the resonances observed in the spectra are normally assigned on the basis of their chemical shifts. An example can be found in Chapter 2. Since in such studies the major parameters that determine the observed chemical shifts are the pH and the complexation by metal ions, the shifts usually correspond closely to those observed for *in vitro* mixtures of similar composition. However, when phosphoproteins are studied, various other factors can contribute to the chemical shifts. This will be considered in more detail later. An advantage of the ^{31}P-NMR method is that usually only very few resonances are present in the spectra since even large macromolecules usually contain only a few phosphoryl moieties. This is in contrast to ^{1}H and ^{13}C-NMR, where the abundance of resonances in a spectrum of a large protein often requires complicated and time consuming experiments to facilitate the assignment. For ^{31}P-NMR spectra, one can usually readily decide which resonance corresponds to what residue. This advantage has probably greatly contributed to the recent popularity of the technique, together with the fact that the ^{31}P-nucleus is virtually 100% abundant and thus expensive enrichment procedures are unneccessry [1]. A disadvantage is the low sensitivity of the nucleus compared to ^{1}H-NMR. Normally this can be offset by using higher frequency instruments, but, as we have shown here, the relaxation of the phosphorus nucleus in systems with longer correlation times becomes dominated by chemical shift anisotropy at higher field strengths resulting in broader lines and a decrease in resolution and signal to noise ratio (Chapters 4 and 9). Application of wide bore magnets and low frequency instruments may be one solution to this dilemma, but in most cases the amount of protein available will be the limiting factor.

As we have shown in Table 8.1 usually the ^{31}P-NMR chemical shift can be readily correlated with the presence of different chemical linkages to a phosphoryl moiety. Ideally, such studies should be performed near pH 8.3, since none of the titration curves of all compounds studied has an inflection in that region (Chapter 8). Table 13.1 is a compilation of the data measured in all ^{31}P-NMR studies of phosphoproteins so far reported. A similar picture to that for the corresponding model compounds emerges from all these studies. The phosphomonoesters SerP and PyrP usually appear between 0 and 9 ppm, and the phosphohistidines are generally found between -4 and -7.5 ppm. To our knowledge no studies have yet appeared on covalent enzyme bound carboxyl phosphates. (The succinyl-phosphate resonance that we observed in Chapter 5 was presumed to be not enzyme bound.) Such residues are expected to give rise to a resonance around -1.5 ppm. Phosphodiester linkages would appear in the same region of the spectrum, but they can usually be readily distinguished since they do not shift with changes of pH even if the protein is fully denatured (Chapter 10). Thus it appears reasonable to identify the nature of a phosphoryl moiety of a phosphoprotein on the basis of the measured chemical shift (see Table 13.1). The only problematic assignment is the differentiation between N-1 and N-3 phosphohistidine residues. Fortunately studies with model compounds have shown that the resonances observed for these residues are separated by 1 ppm [8]. ^{31}P-NMR studies under denaturing conditions have strongly aided in the assignment of N-1 and N-3 hisP residues in HPr and histones [7,8,21].

Although the data presented in Table 13.1 clearly indicate that the resonances observed for certain residues may be in the appropriate range of chemical shift, they are

Table 13.1

^{31}P-NMR parameters measured for phosphoproteins

Protein	Residue	Chemical shift (ppm)[1]	Linewidth (Hz)	Titratable[2]	Frequency (MHz)	Ref.
1. Active site residue						
alkaline phosphatase (E. coli)	SerP	8.5	20	–	36.4	2
		8.5	–		40.5	3
		8.5	20	–	40.5	4
phosphoglucomutase	SerP	3.8	14	–	40.5	5
succ.-CoA synthetase (E. coli)	N3hisP	–4.8	55	N.D.	109.3	6 ch 4,5
HPr E. coli	N1hisP	–4.1	9	–	145.7	7
S. aureus	N1hisP	–4.1	N.D.	–	40.5	8
aspartate transaminase	PyrP	3.7	15[3]	–	40.5	9
serine dehydratase	PyrP	4.0	12	+	72.9	10,11
phosphorylase (rabbit skeletal muscle)	PyrP	3.8/0.6[4]	60/60[4]	–	72.9	12,13
		3.8/0.6[4]	160/70[4]	–	109.3	14,15
phosphorylase (potato)	PyrP	4.5	N.D.	–	72.9	16
phosphorylase (E. coli)	PyrP	3.4	40	+	72.9	17

Table 13.1 contd.

2. Regulatory site residues						
phosphorylase	SerP	3.7/4.8	N.D.	N.D.	72.9	13
		4.2	70	+	109.3	ch 8
myosin light chains	SerP	4.6	40	+	72.9	18
troponin T	SerP	4.5	3	+	72.9	19
ovalbumin	SerP68	5.0	4	+	109.3	ch 9
	SerP344	4.75	2	+	109.3	ch 9
tropomyosin	SerP	4.7	5	+	109.3	ch 8
glutamine synthetase	5'AMP-O-tyr	4.0^5	22	N.D.	40.5	20
histone H_4	N3hisP	$-4.8/-7.3^6$	$9/55^6$	N.D.	81.0	21
3. Residues involved in metal binding						
salivary phosphoproteins	SerP	$4.0/4.5^6$	N.D.	+	145.7	22
casein	SerP	4.5	N.D.	+	40.5	23
phosvitin	SerP	4.5	N.D.	+	40.5	23
		4.5	N.D.	+	109.3	ch 11

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[1] All chemical shifts are given with respect to 85% H_3PO_4, upfield shifts are given a negative sign.

[2] (-) does not shift with pH, (+) shifts with pH, N.D. not determined.

[3] Measured from spectra.

[4] Form III and Form I respectively.

[5] Authors did not specify whether this was upfield or downfield from the reference.

[6] Two different residues.

not always close (e.g. within ± 0.5 ppm) to those observed for standards. Differences in the chemical shift of the order of 0.5 ppm can be caused by differing amounts of hydrogen bonding [24] and or the presence of salt linkages. Our studies on phosvitin (Chapter 11) have shown that each of these may be present but that they do not result in major changes in the ^{31}P-NMR chemical shift, implying that the larger changes must have other causes. For example the resonance for the active site SerP residue of *E. coli* alkaline phosphatase is about 4 ppm downfield from the range of chemical shifts measured for a solution with a SerP standard [2-4]. The N-1 hisP of HPr appears about 1.5 ppm downfield [7,8] and one of the two N-3 hisP residues of histone [21] gives rise to a resonance 2.5 ppm upfield with respect to comparable model compounds [8]. In the case of *E. coli* alkaline phosphatase this shift difference has been attributed to an enzyme-induced bond strain [2-4]. Such a concept seems especially credible in view of the demonstrations that a close correlation exists between bond angles and chemical shifts [25]. Although this may explain some of the unusual shifts observed, other effects may come into play as well. For example, the *E. coli* phosphoryl carrier protein HPr is reported to have a phenylalanine residue close to the hisP [7]. Such a residue could give rise to ring current shifts which could result in the unusual shift. Such phenomena are not unique to the study of phosphoproteins, but are also encountered in ^{31}P-NMR studies of the binding of phosphorylated ligands to enzymes and other proteins. For example, the pyrophosphate moiety of NAD is shifted in different directions upon binding to lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase respectively, although X-ray crystallographic studies suggested that both residues were salt-linked to a positively charged residue [26]. Moreover binding of NADPH to *Lactobacillus casei* and *E. coli* dihydrofolate reductase both gave rise to similar shifts but the binding constants are known to be one hundred-fold different [27-29]. These observations are in keeping with the idea that factors besides the presence of salt linkage must determine the chemical shifts. As indicated in the General Introduction, α-helix dipole moments have been implicated in the binding of phosphoryl moieties to certain enzymes [30]. Possibly these electrical fields could interact with the magnetic dipole of the ^{31}P-nucleus resulting in differences in chemical shift. It is of interest that such fields have been implicated in the binding of NAD(P) nucleotides to lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and *L. casei* dihydrofolate reductase, and in the binding of the FMN phosphoryl moiety in flavodoxin [30]. Notably, for all of these residues, changes in chemical shift upon binding to the protein have been observed [26,27,31]. It would be of interest to investigate this possibility further, since it could be a factor in addition to ring current shifts and bond geometry distortions that could give rise to changes in chemical shift caused by interaction with the protein molecule.

From the foregoing it should be clear that so many factors may contribute to the chemical shifts observed for phosphate groups covalently and uncovalently linked to proteins that deductions to the level of molecular detail on the sole basis of measured chemical shifts are mere speculation. For example, chemical shifts measured for ATP bound to pyruvate kinase have been interpreted as indicative of specific metal complexation [32]. For reasons outlined above and detailed elsewhere [1,33] (Chapter 3), such practice should be strongly discouraged. Moreover many authors have assumed that since the chemical shift of a protein bound phosphomonoester species are usually closest to those observed for a dianionic standard solution, that bound phosphomonoesters are themselves always dianionic [9,10,12-19]. Although most of these deductions, especially for the titratable residues, are likely to be correct (see section on titration studies), all of these conclusions are based on "... the questionable assumption that the chemical shift in the bound form is identical to that of the corresponding ionic species in the unbound form ...." [1].

One other point must be mentioned here. Although it is tempting to think that all phosphorus residues observed in the spectra are covalently linked to the protein, this is not necessarily the case as we have observed in Chapter 10.

Also, studies on myosin light chains indicated the presence of various noncovalently linked phosphorus-containing impurities [18]. Thus although the presence of a phosphodiester linkage deduced from ^{31}P-NMR for glucose oxidase and flavodoxin may represent a bridged phosphoryl moiety analogous to a disulfide linkage [31,34], such a possibility remains highly speculative and awaits confirmation by identification of such a complex by chemical means.

13.2 Interpretation of linewidths measured in ^{31}P-NMR spectra

As we have indicated in Chapters 4 and 9, the linewidths can be readily analyzed when frequency-dependent measurements are performed. This approach allows for a separation of the field-independent and field-dependent terms. Field-independent terms are mainly caused by proton-phosphorus dipole-dipole contributions. In the case of *E. coli* succinyl-CoA synthetase our analysis has led to the suggestion that the covalent hisP residue is protonated to the monoanion form. This experiment should be repeated with varying ratios of H_2O and D_2O in the buffer. Such experiments could provide further support for this conclusion. It is of interest that not one of the model compounds studied (Chapter 8, Table 8.2) has a pKa consistent with protonation to a monoanion at physiological pH. One would expect the pKa of hisP to resemble those of arginine or creatine phosphate (pKa = 4.25). However, others have suggested that the pKa <3.5 [35] or that it is in the range pKa = 8-9 [8]. Thus it is not clear at present whether the model compound is protonated to the monoanionic form at pH 7.2, or if this occurs only in the active site of *E. coli* succinyl-CoA synthetase. Analysis of the field-independent term for the SerP residue of glycogen phosphorylase *a* has indicated that this residue is in a dianionic form (Chapter 4). This conclusion is further supported by its titration behaviour (Chapter 8) and its chemical shift which is close to that of an unbound dianionic species.

The field-dependent terms are composed of contributions of chemical shift anisotropy to the relaxation as well as a possible term caused by field dependence of a difference in chemical shift for two exchanging conformations [36]. Thus only when the resonance is that of a single conformation is the field-dependent contribution a valid measure of the CSA contribution, and only then can the rotational correlation times be calculated. Alternatively, if the rotational correlation times are known the anisotropy term can be calculated and subsequently compared to that measured for model compounds. The first approach has led us to conclude that the active site hisP residues of succinyl-CoA synthetase and HPr are immobilized (Chapter 4). Others have shown that the active site SerP residue of phosphoglucomutase is immobilized as well [5]. Moreover we have calculated using the measured linewidths from Table 13.1 and the rotational correlation times measured by others [36,37] that the anisotropy terms for the active site SerP of *E. coli* alkaline phosphatase and the PyrP of aspartate transaminase, serine dehydratase and glycogen phosphorylase form I and form III, all exceed 300 ppm. Since Table 4.2 indicates that the factor expected for a phosphomonoester is usually 120 ppm, it appears that in all of these cases exchange broadening contributes to the field dependent term as well.[1] Thus it appears that all these residues are in the fast to intermediate exchange limit between two different bound conformations. Further studies including analysis of the frequency dependence of the linewidths are necessary to indicate if this "wobbling" indeed occurs in the active sites of these enzymes. All the foregoing indicates that the active site phosphoryl moieties are immobilized or that they are in exchange between two different bound conformations. As seen in Chapter 5, this exchange can sometimes be induced by the addition of other substrates (addition of CoA induced the exchange between two different conformations for the phosphohistidine). In contrast to such studies on active site residues, our results have suggested mobility in the regulatory sites that are phosphorylated and dephosphorylated by protein kinases and phosphatases. Most of these residues are in the fast exchange limit with a calculated rotational correlation time lower than that of the protein molecule. Only for glycogen phosphorylase *a* have we observed that the residue was in exchange between a salt linked and a solvent exposed conformation (Fig. 8.3).

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[1] One of the possibilities considered to explain the increase in the anisotropy term upon binding to a protein is that of bond strain (see Chapter 4). It should be pointed out here that if the anisotropy term increases as a result of bond strain, one would expect to see changes in the chemical shift as well. This is only observed for *E. coli* alkaline phosphatase [2-4].

Other groups have also attempted to interpret the linewidths. For example, the German group (Feldmann, Hull, and Helmreich) have considered the PyrP (form I) of rabbit skeletal muscle glycogen phosphorylase to be immobilized and to be in only one conformation. Linewidths for all other proteins studied by this group were measured at the same instrument frequency and were directly compared to phosphorylase after adjustments were made for differences in molecular weight [10,13,16-19]. We feel that this approach may give rise to tenuous conclusions about the mobility of the residues, firstly because it is not always clear whether the phosphorylase is in the tetrameric or dimeric form in the conditions of the experiment, and secondly, since our calculations have suggested that the observed resonance may represent more than one conformation. Moreover, the field-dependent and field-independent terms may contribute in different proportions for each of proteins under study. In a recent study at 81 MHz of histone H_4 the linewidths (9 and 55 Hz) of resonances for two hisP residues have been interpreted in terms of mobility [21] (the narrow resonance as mobile and the broad resonance as immobile). These linewidths may be directly compared to that of *E. coli* HPr (9 Hz at 145.7 MHz) since the molecular weights of histone H_4 and HPr are similar. The hisP residue on HPr is immobile (Chapter 4), thus implying that the narrow histone H_4 resonance represents an immobilized residue and that the broad resonance is that of a severely exchange broadened hisP residue. These considerations have strong implications for the model proposed by Fujitaki *et al.* [21].

13.3 Interpretation of the ^{31}P-NMR pH titration studies

X-ray crystallography [38] and chemical modification studies [39] have often indicated that bidentate guanidino groups of arginine residues can interact with phosphomonoester moieties of ligands or regulatory SerP residues. If such a linkage is very strong one would expect that changes in pH would not effect the chemical shift observed in the ^{31}P-NMR spectra for such bound species. This predicted behaviour has indeed been observed for the 2'-phosphoryl moiety of NADPH or NADP$^+$ bound to *L. casei* or *E. coli* dihydrofolate reductase [27,29]. These enzymes have a high specificity for NADPH as a coenzyme over NADH [28] suggesting that the 2'-phosphoryl moiety plays a crucial role in the binding of the nucleotide. Our studies on ligand binding to ATP-citrate lyase support the idea that phosphoryl moieties on the 2' and 3' position of the ribose ring serve as recognition and discrimination points for binding. Neither NADH nor dephospho CoA, nor cyclic-2'3' compounds could substitute for the protective effect exerted by either nucleotide.[2,3] In our studies with ATP-citrate lyase, higher concentrations of "fragments" of the coenzymes could substitute to give the same effects, suggesting that these bind to the same site but with a higher Kd (mM). Similarly, 2'AMP can occupy the NADPH binding site of *L. casei* dihydrofolate reductase. Its Kd is about ten-fold higher than that observed for NADP$^+$. However, when ^{31}P-NMR pH titration studies were performed with 2'AMP bound to this enzyme the resonance shifted with pH [28], whereas the 2'-phosphoryl resonance of NADPH or NADP$^+$ did not shift when enzyme-bound [27,29]. These studies provide a framework for the following interpretation of the ^{31}P-NMR pH titration studies on regulatory SerP residues. In all cases studied so far (ovalbumin, glycogen phosphorylase *a*, tropomyosin, phosvitin, troponin T [19] and myosin light chains [18]) it has been shown that these residues can be titrated, giving rise to pH-dependent shifts in their respective ^{31}P-NMR spectra. Thus it might be argued that if a salt linkage exists between the SerP residue and positively charged amino acid side chains in any of these proteins, the strength of this binding must be lower than that of NADP$^+$ bound to *L. casei* dihydrofolate reductase (Kd = 22 μM) and is presumably comparable to that

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[2] Not all enzymes have an absolute requirement for this recognition. Dephospho CoA is a reasonable substrate for *E. coli* succinyl-CoA synthetase, for example [40].

[3] We have not analysed the binding of these nucleotides by ^{31}P-NMR since the resonances of the 2' and 3' phosphoryl moieties overlap in the spectra with that of the regulatory SerP.

of 2'AMP bound to this enzyme (270 μM). On this basis, the fact that all regulatory SerP residues can be titrated suggests that their respective "Kd"s for the interaction between the phosphoryl moiety and a positively charged amino acid side chain must be larger than 100 μM (*i.e.*, ΔG° approx. -5.5 kcal).

We have indicated in Chapter 8 that two different models have been proposed to explain how regulatory phosphorylation sites may exert their function. In the first model the dianionic serine phosphate residue is salt linked to either arginine or lysine residues and in doing so may help stabilize one specific protein conformation [38,41]. In the second model a mobile phosphoryl group may prevent an interaction between protein domains [18,19]. In the latter case one would expect the resonance to show titration behaviour with a Hill coefficient similar to that of standards (see Chapter 8). When salt linkages are involved these have to be weaker than Kd = 100 μM to allow for a normal titration behaviour, but one would expect to see higher Hill coefficients ($n>1$) when positive charges are proximal (Chapter 8). Moreover, the fairly normal titration behaviour observed for all regulatory SerP residues may be taken as an indication that the bound form is indeed a dianionic species as was already suggested by the similarity in chemical shift to solution dianionic SerP standards.

Another point of interest should be mentioned here. As can be seen in Table 13.1 many active site phosphoryl residues (*i.e.*, catalytic intermediates) do not show changes in the chemical shifts of their resonances upon changes in pH. The only exceptions reported are serine dehydratase and *E. coli* phosphophorylase. Most workers have interpreted such behaviour to indicate that the given active site phosphoryl moiety is not exposed to solvent. However, it could be argued that the residue could be exposed to solvent but that it is involved in a salt linkage (e.g., with an active site arginine residue) that is sufficiently stable to block its titration ("Kd" < 100 μM).

13.4 Reversible phosphorylation

Our studies have clearly shown that the large majority of SerP regulatory sites is mobile and solvent exposed at least part of the time. This mobility and surface location may be a necessary requirement for protein kinases and phosphatases to have access to the site in order to be able to transmit the hormonal stimulus. Further support for this idea comes from our studies on ovalbumin. The SerP68 moiety is located near the surface of this protein, but it has very little residual mobility with respect to the protein surface and it is also insensitive to phosphatase digestions (Chapter 9). Thus although a regulatory SerP site may be involved in a salt linkage to stabilize a specific conformation and hence exert its function, we know now that the "Kd" for that linkage is higher than 100 μM and thus it is exposed to solvent part of the time so it is available to phosphatases. It is perhaps surprising that such a relatively weak interaction can result in dramatic changes in the activity of an enzyme such as for glycogen phosphorylase *a* [38,42].[4] The alternative model, with a mobile dianionic phosphoryl moiety preventing interaction between protein domains, may play a role in certain systems as well. In the course of our studies on glycogen phosphorylase *a*, tropomyosin and phosvitin we have seen evidence that interactions with other amino acids may exist, whereas in ovalbumin no evidence for such interactions could be found. It is possible that in the last case the two phosphates may prevent an interaction with other proteins, especially since they constitute a major contribution to the surface charge of this protein (Chapter 9). Thus these results are compatible with a biological role for both proposed models.

13.5 Phosphoryl transferring enzymes

Our studies on *E. coli* succinyl-CoA synthetase have provided some intriguing insights into the complex mechanism of this enzyme. First of all, we have demonstrated that the hisP residue is immobile (Chapter 4), a property that may facilitate in-line nucleophilic attack at the phosphoryl moiety [43]. Moreover, evidence has been obtained that binding of CoA and phosphorylation by ATP of the second active site provide for a modulation of the phosphoryl transfer from the histidine onto the succinate (Chapter 5). Our detailed analysis of linewidth has allowed us to interpret the CoA-induced broadening of the hisP resonance in molecular terms suggesting that the phosphohistidine can exchange (in the intermediate to slow exchange limit) between two conformations (see Fig. 5.9. The ATP modulation observed for the second partial reaction has been interpreted in terms of catalytic cooperativity between the two active sites. This concept has gained further support from our studies of nitrate inhibition and of the hybrid enzymes containing inactive modified subunits. Since it has been shown that both active sites are available for catalysis in the native enzyme, we favour an alternating site model (see Fig. 5.10) over other models proposed (Chapter 6). A similar model has been proposed in the past for other enzymes, most notably malate dehydrogenase [44] and *E. coli* alkaline phosphatase [45]. Although this idea has not been challenged for the first enzyme, the concept of alternating sites catalytic cooperativity in alkaline phosphatase has recently endured severe criticism. Since this enzyme has a dimeric structure comparable to the $(\alpha\beta)_2$ dimer of dimers structure of *E. coli* succinyl-CoA synthetase a brief discussion of some of the alkaline phosphatase literature may be illustrative.

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[4] Our titration studies on phosphorylase *a* were performed in the presence of glucose which is known to facilitate the action of the phosphatase. Possibly the interaction in the absence of glucose could be much stronger. Unfortunately this cannot be experimentally verified; in the absence of glucose the SerP and the PyrP resonance are overlapping.

The proposals that the mechanism of action of alkaline phosphatase involves alternating sites catalytic cooperativity find their origin in the half-of-the-sites reactivity and strong negative cooperativity observed for this enzyme [45]. Since it was discovered in 1962 that the enzyme could be dissociated and reconstituted at low pH [46], many studies have focussed on the use of hybrids. It was first demonstrated that such hybrids, having partial enzyme activity, could be formed by mixing native active subunits and mutationally altered but antigenically related inactive subunits [47,48]. Subsequently hybrids were purified by making use of density (D,^{15}N) labeled subunits [49] whose purity could be confirmed by radioactive specific activity determinations. It was shown that no subunit interchange occurs between hybrid dimers [50] and kinetic studies on such a purified hybrid consisting of one active subunit and one inactive subunit showed that all transient and steady state rates observed for the hybrid are half those seen for the wild type, suggesting that both active sites act independently [51]. Subsequent studies with hybrids containing chemically modified inactive subunits have provided further evidence for the independence of both active sites [52,53]. However, recent experiments with immobilized renatured subunits, constructed according to the methods of Chan [54], have indicated that the separated but refolded monomer is inactive [55], suggesting that some form of subunit interaction is a requirement for catalytic activity. Recent kinetic experiments have, however, cast doubt on alternate functioning of subunits [56,57] and ^{113}Cd-NMR experiments have suggested that the half-of-the-sites reactivity prevails only when not all of the metal binding sites are saturated [58]. To summarize, it appears that subunit interactions are necessary to provide a conformation which is active, but if sufficient metal ion is present the two active sites act independently. This model would provide a rationale for the subunit structure of alkaline phosphatase. In contrast aldolase, for example, is active as a monomer and is equally active but more stable in higher aggregated forms [54]. Glyceraldehyde-3-phosphate dehydrogenase appears to be more active as a monomer than as a tetramer [59] and a similar situation may exist for *E. coli* succinyl-CoA synthetase [60]. Thus the rationale for the subunit structure may be different in each case. Obviously there are various examples that demonstrate an effect of subunit interaction on the specific activity of enzymes. Our data presented in Chapter 5 and 6 strongly suggest that both active sites in *E. coli* succinyl-CoA synthetase do not act independently. The validity of the model that we present (Fig. 5.10) might be further examined by further experiments employing purified hybrids similar to those outlined above for alkaline phosphatase, or single turnover experiments. Spectroscopic studies of the cooperativity of conformational changes upon substrate binding (as performed for other enzymes with strong negative cooperativity [61,62]) or studies with immobilized renatured subunits could provide further tests for alternating sites catalytic cooperativity as an inherent property of *E. coli* succinyl-CoA synthetase. Such a property may have implications to other phosphoryl transferring enzymes. It is encouraging in this respect that *E. coli* glutamine synthetase, an enzyme, like succinyl-CoA synthetase, may proceed via a noncovalent acyl phosphate intermediate, has recently been proposed to function in a catalytic cooperative way [63,64]. Despite this, others [65] have alleged that alternating sites cooperativity is only a test-tube phenomenon and does not contribute to the catalytic efficacy of enzymes *in vivo*.

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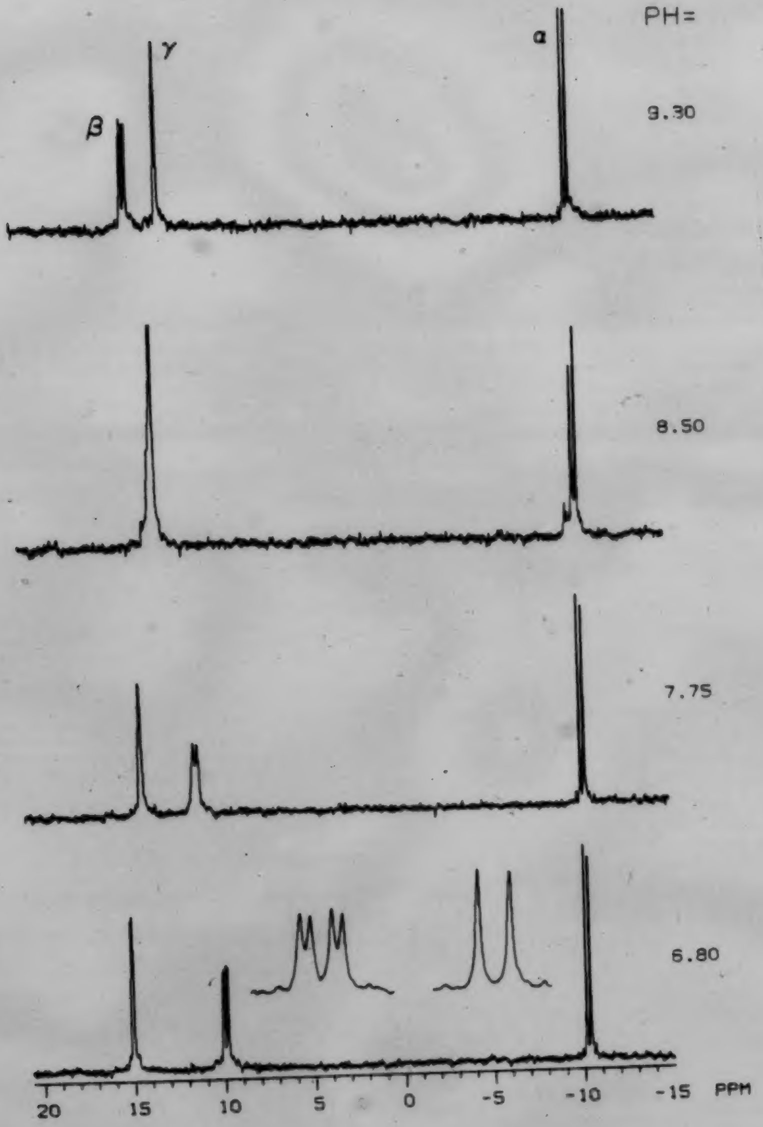
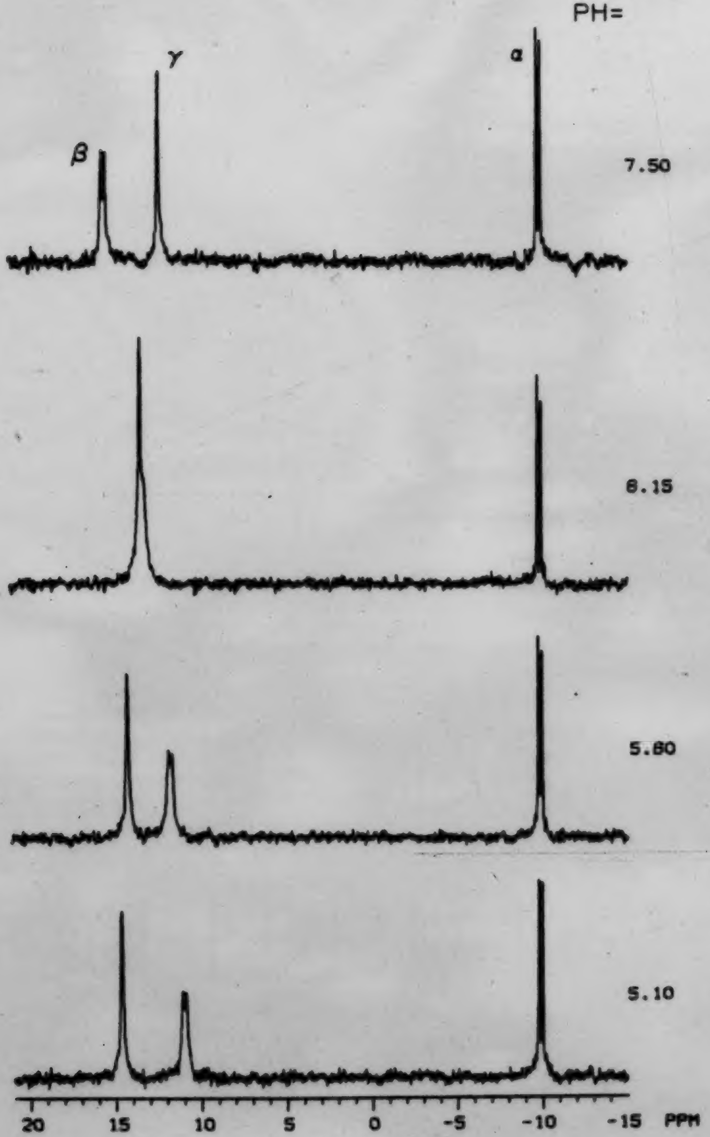
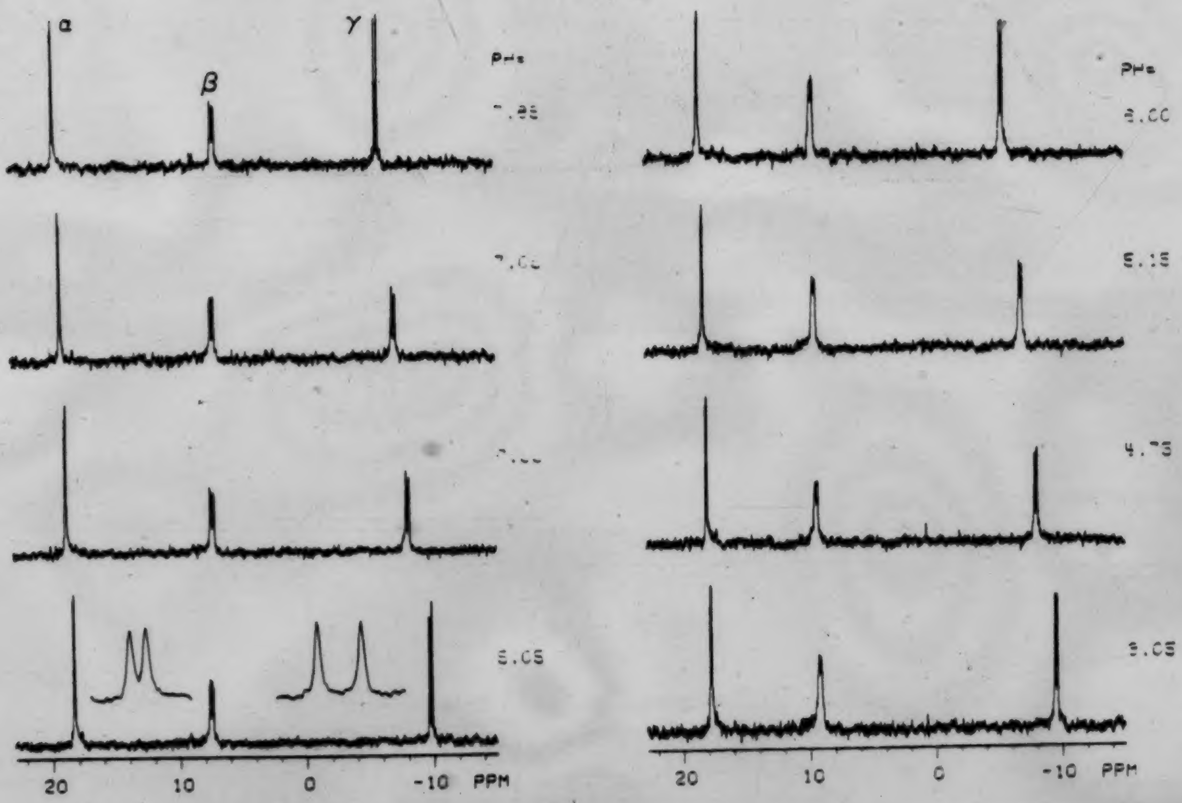
Appendix 1



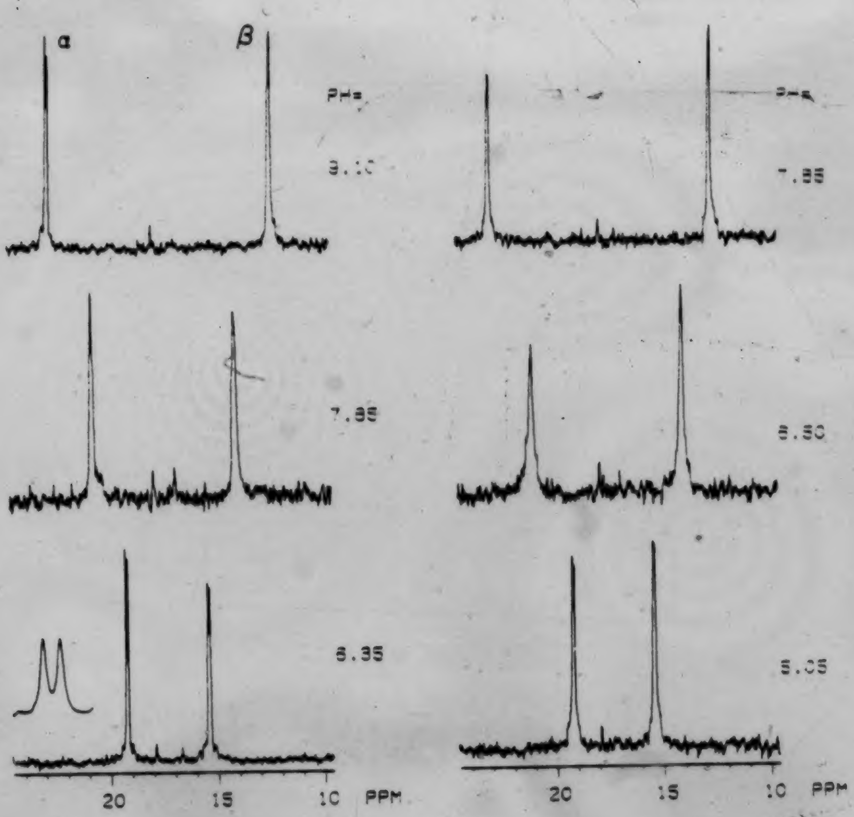
App. 1.1 ^{31}P-NMR pH titration study of AMPPCP (see Chapter 3, Fig. 3.2).



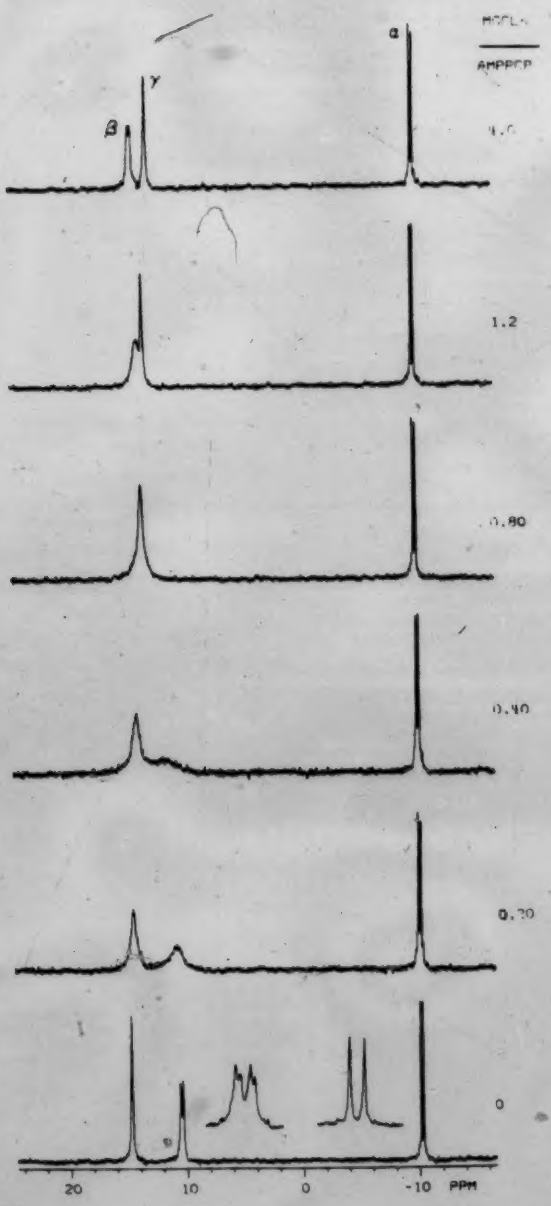
App. 1.2 ^{31}P-NMR pH titration study of Mg AMPPCP (see Chapter 3, Fig. 3.3). The $MgCl_2$ to AMPPCP ratio was 5:1.



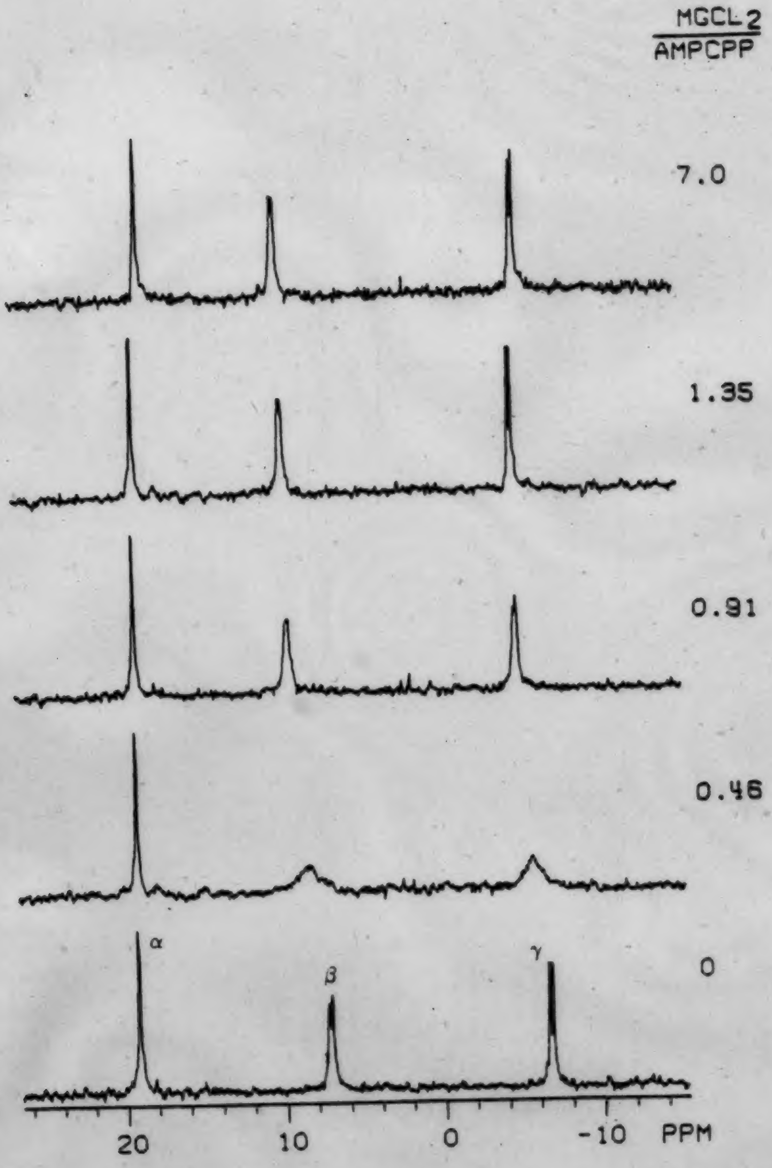
App. 1.3 ^{31}P-NMR pH titration study of AMPCPP and MgAMPCPP (see Chapter 3, Fig. 3.3). The $MgCl_2$ to AMPCPP ratio was 5:1.



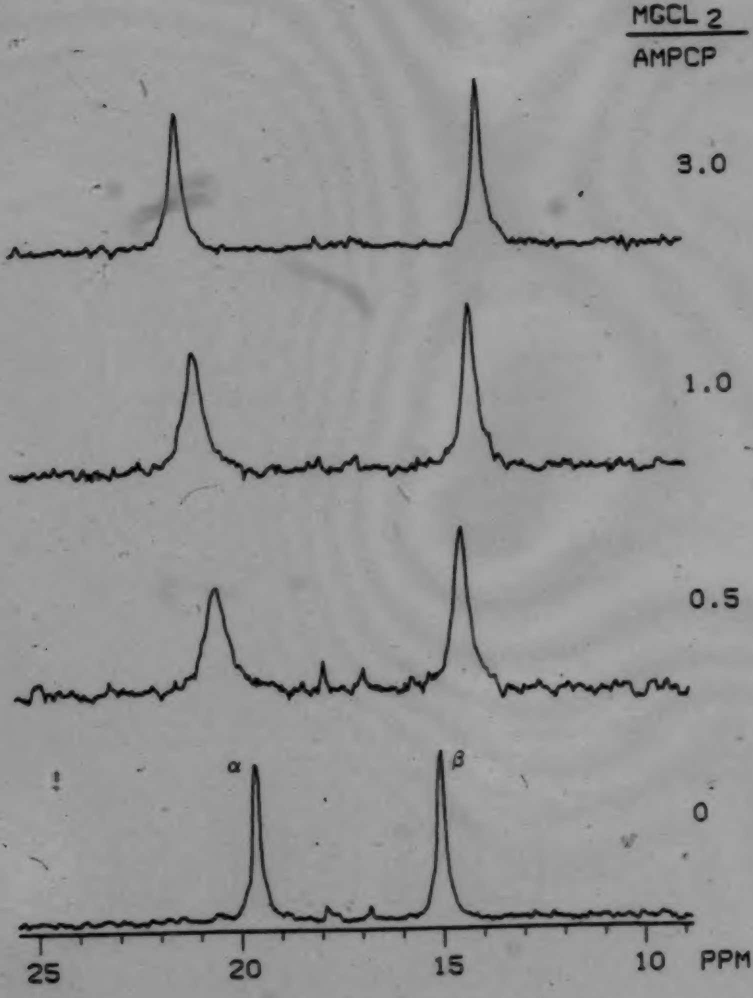
App. 1.4 ^{31}P-NMR pH titration study of AMPCP and MgAMPCP (see Chapter 3, Fig. 3.4). The $MgCl_2$ to AMPCP ratio was 5:1.



App. 1.5 ^{31}P-NMR Mg^{2+} titration study of AMPPCP (pH 8.3) (see Chapter 3, Fig. 3.5).

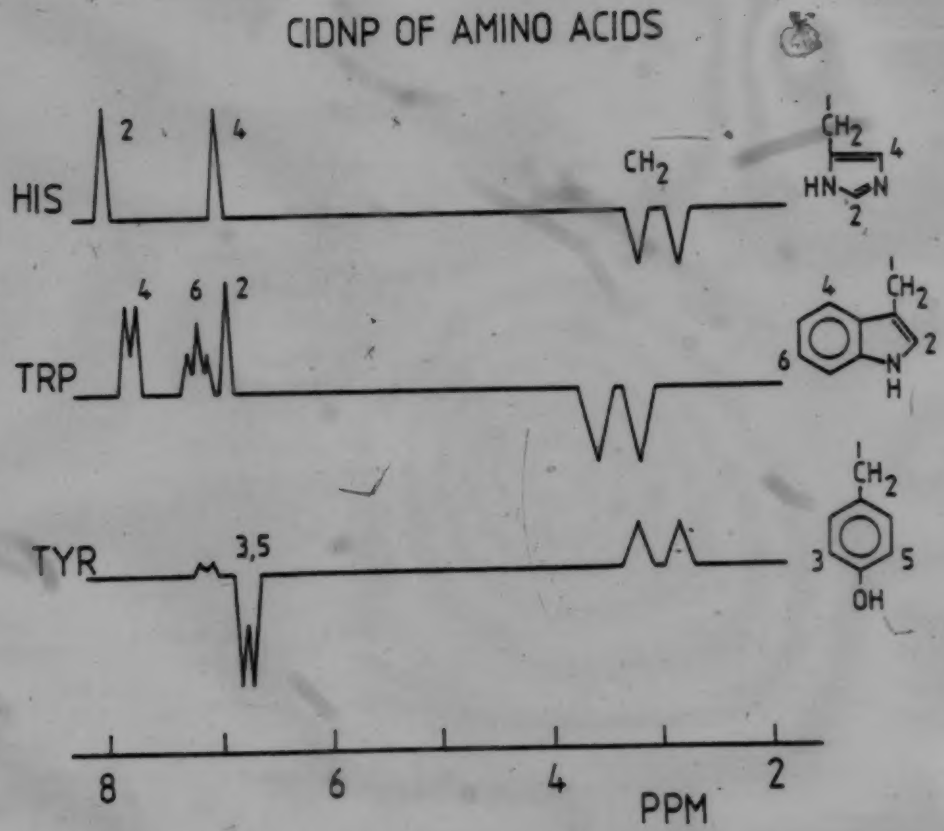


App. 1.6 ^{31}P-NMR Mg^{2+} titration study of AMPCPP (pH 8.3) (see Chapter 3, Fig. 3.6).

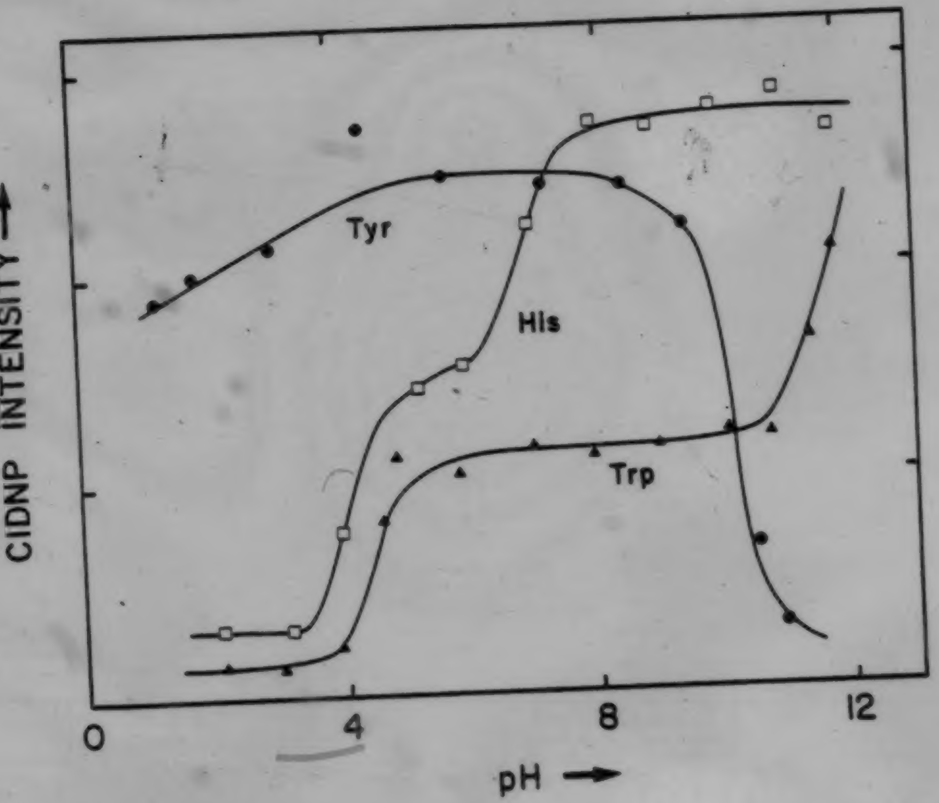


App. 1.7 ^{31}P-NMR Mg^{2+} titration study of AMPCP (pH 8.3) (see Chapter 3, Fig. 3.7).

Appendix 2



App. 2.1 Schematic representation of the flavin induced 'H photo-CIDNP effects observed for the amino acids histidine, tryptophan and tyrosine. This figure is taken from R. Kaptein in Biological Magnetic Resonance, Vol. IV, Eds. L.J. Berliner and J. Reuben, Plenum Publishing Co., New York. (In Press).



App. 2.2 pH dependence of the photo-CIDNP effect of the amino acids histidine, tryptophan and tyrosine. This figure is taken from R. Kaptein in Biological Magnetic Resonance, Vol. IV, Eds. L.J. Berliner and J. Reuben, Plenum Publishing Co., New York. (In Press).